

Metallo- β -lactamase Mediated Carbapenem Resistance in Gram Negative Bacilli

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Abstract.

Carbapenem antibiotics were thought to be the ultimate β -lactams, combining the action of a β -lactam with that of a β -lactamase inhibitor in a single molecule. These compounds are very effective antimicrobials and are currently licensed for use as last line treatments in Intensive Therapy Units (ITUs) and remain active against organisms producing extended spectrum TEM- and SHV- derived enzymes which are able to hydrolyse third generation cephalosporins.

In this thesis a study of imipenem resistant *Bacteroides fragilis* clinical isolates from several sources including the Public Health Laboratory Service in Cardiff and the Scottish Anaerobe Reference Laboratory was undertaken. Three of these isolates were found to have Minimum Inhibitory Concentrations (MIC's) of imipenem greater than the breakpoint set by the NCCLS of 16mg/l. In all cases, the isolates were found to produce an imipenem hydrolysing β -lactamase. Biochemical analysis has shown that these strains each produce a single β -lactamase with a substrate and inhibitor profile similar to that of the CfiA metallo- β -lactamase previously described from a *Bacteroides fragilis* isolate.

Whilst attempting to optimise the assay conditions for these enzymes, it was discovered that the concentration of zinc sulphate present during the preparation procedure and during the assay significantly affected the specific activity and substrate profile of these enzymes. By splitting a single culture of a metallo- β -lactamase producing strain, it was possible to demonstrate that this effect was a direct result of the zinc sulphate concentration present during the preparation and assay of these enzymes. It was noted that these enzymes were inhibited by the presence of excess zinc sulphate, and that this inhibition was reversible and substrate specific, affecting carbapenem hydrolysis more than any other substrate. This observation was found to extend to several other metallo- β -lactamases.

A survey of *Bacteroides* clinical isolates from the Edinburgh Royal Infirmary was also undertaken to determine the frequency of resistance to several antimicrobials. The rate of imipenem resistance was found to be 0.6% ($n = 166$).

Imipenem resistant isolates from several species including *Burkholderia*, *Enterobacter* and *Stenotrophomonas* were examined for carbapenemase production. Only the *Stenotrophomonas* isolates were found to produce a β -lactamase capable of hydrolysing imipenem.

Ten clinical isolates from the genus *Flavobacterium* were examined for carbapenemase production. All isolates were found to hydrolyse imipenem, although in three isolates, the rate was very low. The addition of the metal ion chelator EDTA was found to inhibit both the nitrocephin and imipenem hydrolysis of all but two of these isolates. Two strains were also identified each of which produced a novel metallo- β -lactamase, and the β -lactamases of one of these (*Flavobacterium spiritivorum* NCTC 11388) were characterised in greater detail.

Declaration.

The experiments and composition of this thesis are the work of the author unless otherwise stated.

J.G.R. Munn

9th October 1995

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I would like to thank my mother, Mrs. J. H. Huxley, for her help and encouragement in the preparation of this book. I would also like to thank my father, Mr. J. H. Huxley, for his help and encouragement in the preparation of this book. I would also like to thank my mother, Mrs. J. H. Huxley, for her help and encouragement in the preparation of this book.

The known is finite, the unknown infinite; intellectually
we stand on an islet in the midst of an illimitable
ocean of inexplicability.

T.H. HUXLEY, 1825-1895

Thanks are also due to my mother, Mrs. J. H. Huxley, and her husband, Mr. J. H. Huxley, for the use of their collection.

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Abbreviations.

6-APA	6-aminopenicillanic acid
7-ACA	7-aminocephalosporanic acid
A _x	Absorbance at x nm
amox	amoxycillin
amp	ampicillin
BS	broad spectrum
BSAC	British Society for Antimicrobial Chemotherapy
carb	carbapenems
cefep	cefepime
ceph	cephalosporins
CF	cystic fibrosis
cfu	colony forming units
cfx	cefuroxime
cip	ciprofloxacin
clav	clavulanic acid
cld	cephaloridine
clox	cloxacillin
ctx	cefotaxime
czd	ceftazidime
Da	daltons
DM	Davis and Mingioli minimal salts solution
DST	diagnostic sensitivity agar
EDTA	ethylenediaminetetraacetate
g	acceleration due to gravity
GBA	gentamicin blood agar
FPLC	fast protein liquid chromatography
IEF	iso-electric focusing
imp	imipenem
IST	isosensitest
ITU	intensive therapy unit
λ	wavelength of light
mero	meropenem
MIC	minimum inhibitory concentration
NCCLS	National Committee for Clinical Laboratory Standards
NCFN	nitrocephin
NCTC	National Collection of Type Cultures
NS	narrow spectrum
PAGE	polyacrylamide gel electrophoresis
PBP	penicillin binding protein
pen	penicillins
PHLS	Public Health Laboratory Service
pI	iso-electric point
PIPES	piperazine-N,N'-bis[2-ethanesulphonic acid]
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
Tn	transposon
Tris	tris[Hydroxymethyl]aminomethane]
V	volts
W	watts

Glossary.

Basal Activity [Chapter 5]	The rate of hydrolysis of substrate by the tested β -lactamase in the presence of no ZnSO_4 supplement. This term relates only to data presented in Chapter 5.
BFR_0 BFR_1 BFR_{1000} [Chapter 5]	The subscripts 0, 1 and 1000 denote the concentration of zinc sulphate (in μM) present during the β -lactamase preparation, in this case BFR (<i>Bact. fragilis</i>).
ID_{50}	Concentration of inhibitor required to inhibit hydrolytic activity by 50%.
MIC_{50} [Chapter 4]	Minimum inhibitory concentration at which 50% of the tested isolates were found to be sensitive.
MIC_{90} [Chapter 4]	Minimum inhibitory concentration at which 90% of the tested isolates were found to be sensitive.

Chapters in brackets denote the first occurrence of each term.

All other terms are standard scientific notation and are quoted in standard units.

Publications and Presentations.

Publications.

Munn, J.G.R., Thomson C.J., and Amyes S.G.B. (1994) "Effect of zinc on the imipenemase activity of a metallo- β -lactamase produced by a *Bacteroides fragilis* clinical isolate." *Journal of Antimicrobial Chemotherapy* 34 295-297.

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CHAPTER ONE

Introduction.

Few developments in the history of medicine have had such a profound effect upon man and society as the development of the power to control infections due to micro-organisms. Developed countries have been able to control and treat many bacterial infections which previously devastated populations. The threat of death from infection has diminished to such an extent that it is now the exception, rather than the expected outcome of a diagnosed infection.

1.1. Antimicrobial Agents: A Brief History.

The use of poultices and tinctures for the treatment of infections and general malaise have been documented for thousands of years. These remedies were passed down from generation to generation and improved over the centuries. Many of these treatments can be shown to contain compounds which give them a sound pharmacological basis, although the reason for activity was not necessarily understood at the time.

Mercuric chloride was used by Arabian physicians in the middle ages for preventing sepsis on open wounds. Antiseptics came into general medical use in the nineteenth century, although these early attempts were not generally accepted until Pasteur's publication in 1863 of the microbial origin of putrefaction. The problem was to discover compounds having a selective action against the microbial cell compared with the cells of the host animal.

Following the success by Lindgard in the treatment of trypanosomiasis with arsenous oxide, Ehrlich attempted to make other related arsenicals which were less toxic. He found that these compounds were active against the causative agent of syphilis, and in 1907 he discovered salvarsan which soon

became the standard treatment for syphilis. This was later coupled with bismuth therapy, and was used until superseded by penicillin in 1945. Ehrlich was probably the first person to encounter the problem of resistance to chemotherapeutic compounds by the observation that trypanosomes could become resistant to certain treatments, and that this resistance extended to chemically related compounds, but that there was no cross resistance between compound groups [1,2].

Other attempts at producing antimicrobials were unsuccessful and no practical progress was made until 1935 when Domagk reported the activity of Prontosil rubrum against animal infections. Trefouel showed that Prontosil was metabolised in the body to sulphanilamide, the active antibacterial compound. This was the first sulphonamide identified, and their development would have been more extensive had it not been for the advent of penicillin and other antibiotics so soon afterwards. The only synthetic compounds widely used apart from sulphonamides are trimethoprim, the quinolones and metronidazole [1].

1.2. The Penicillins.

1.2.1. The Discovery of Penicillin.

In 1929 Fleming reported that a mould growth contaminating one of his plates seeded with *Staphylococcus aureus* was able to inhibit the growth of this bacterium. This mould was identified as *Penicillium notatum*. He later showed that this mould produced a freely diffusible substance which he named penicillin. This compound was highly active against Gram positive bacteria, and he found that when injected into animals, broth containing this compound was no more toxic than broth alone. Unfortunately this mould did not produce penicillin in sufficient quantities for the drug to be successfully purified and tested, and little work was conducted on penicillin until 1940. In this year, Florey and Chain re-examined the mould described by Fleming. They succeeded in producing a solid preparation containing partially purified penicillin [3,4]. Large scale fermentation techniques and

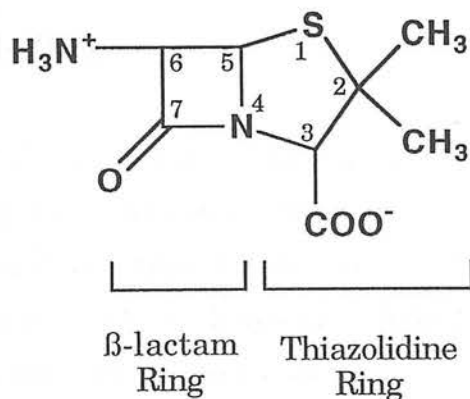
the later discovery of *Penicillium chrysogenum* which produced penicillin in significantly greater quantities than *Penicillium notatum* allowed large scale penicillin production to become both practical and affordable.

It was found that the addition of various adjuncts to the fermentation medium could determine the side chain added to the penam nucleus and thereby the β -lactam produced would be predominantly a single compound [5]. Benzylpenicillin (Penicillin G) was produced by the addition of phenylacetic acid to the fermentation medium. Similarly, the addition of phenoxyacetic acid led to the production of the first penicillin variant, phenoxymethylpenicillin (Penicillin V) [6]. This compound had significantly increased acid stability which led to a decreased loss of the active compound owing to the low pH of the stomach when given orally.

1.2.2. 6-Aminopenicillanic Acid.

The elucidation of the structure of 6-aminopenicillanic acid (6-APA) [7] facilitated the formation of many semi-synthetic penicillin derivatives with varying spectra of activity. 6-APA is the condensation reaction product of alanine and β -dimethylcysteine and is shown in Figure 1.1 below.

Figure 1.1: Structure of 6-Aminopenicillanic Acid.



1.2.3. Classification of Penicillins.

Modification of the acyl side-chain of 6-APA results in the steric protection of the β -lactam ring and thus protects it from attack and hydrolysis by several β -lactamase enzymes whilst also defining its spectrum of activity and pharmacokinetic properties. Many such modifications have been made, and examples of the resulting compounds are shown in Table 1.1 below.

Table 1.1: Classification System for Penicillins.

Penicillin Group			
Natural Penicillins	Anti Staphylococcal Penicillins	Broad Spectrum Penicillins	Compounds active against <i>P. aeruginosa</i>
Benzylpenicillin (G) Phenoxymethylpenicillin (V)	Methicillin Nafcillin (isoxazolyl-) Cloxacillin Dicloxacillin Flucloxacillin Oxacillin	(amino-) Ampicillin [†] Amoxycillin Ciclacillin Amdinocillin	Temocillin (acylureido-) Azlocillin Mezlocillin Piperacillin (carboxy-) Carbenicillin Ticarcillin

[†] and derivatives.

Adapted from [8].

(*italics*) denote the structural family of the compounds listed.

1.2.3.1. Natural Penicillins.

These compounds exhibited activity against almost all Gram positive organisms, Gram negative cocci and some Gram negative bacilli. These compounds rapidly penetrate most tissues but are hydrolysed by virtually all β -lactamases. However, these drugs are often first choice therapy for streptococcal and pneumococcal infections.

1.2.3.2. Anti Staphylococcal Penicillins.

Anti staphylococcal penicillins are penicillin derivatives that are penicillinase resistant. These drugs are first line treatment for staphylococcal infections. However, the emergence of methicillin resistant *Staphylococcus aureus* (MRSA) in the early 1980s has eroded their usefulness.

1.2.3.3. Broad Spectrum Penicillins.

These penicillins were the first compounds with bactericidal activity against *Escherichia coli* and *Haemophilus influenzae*, and yet retained activity against penicillin sensitive Gram positive bacteria.

1.2.3.4. Penicillins Active Against *P. aeruginosa*.

These compounds possess an antibacterial spectrum of activity similar to ampicillin with activity against several species of Enterobacter. The carboxypenicillins must be given by the intramuscular or intravenous route as they are not absorbed after oral administration.

1.2.4. Penicillin Use In The 1990s.

Structural modifications of the β -lactam acyl side chains as discussed earlier have gone some way to increasing β -lactamase stability, but bacteria have countered this by producing enzymes with a much broader spectrum of activity. The increasing spread of bacterial resistance, particularly amongst the *Enterobacteriaceae* and *H. influenzae*, has curtailed the usefulness of such drugs in these clinical settings. However, these drugs are still prescribed today for minor infections, more than 50 years after their discovery.

β -lactam compounds are generally non-toxic to humans, although immunological sensitisation has been observed in a small proportion (<10%) of those treated with these compounds. This sensitisation precludes the use of these compounds in those individuals affected because of the risk of anaphylaxis, although some cephalosporins cause no reaction in some patients sensitive to penicillins [9].

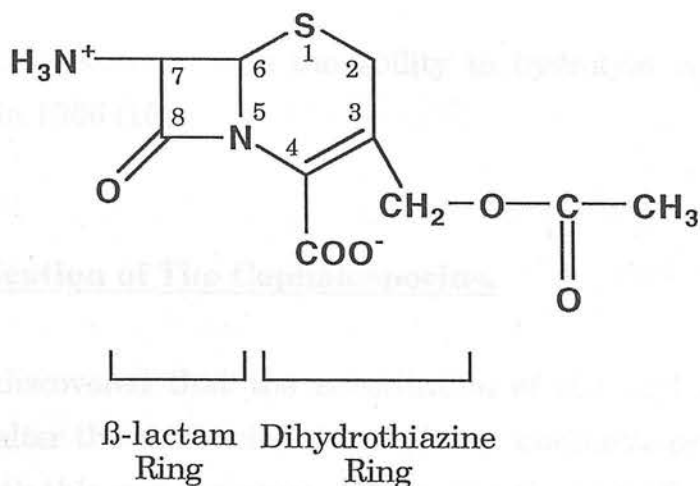
1.3. The Cephalosporins.

1.3.1. The Discovery of the Cephalosporins.

The first report of a cephalosporin was made by Brotzu who isolated a strain of *Cephalosporium acremonium* from a sewage outfall in Sardinia in 1945. Treatment of bacterial infections and abscesses with a crude extract of this mould gave promising results and so, in 1948 he sent a sample of the mould to Florey in Oxford. A series of molecules were isolated from this mould which exhibited bactericidal activity against Gram positive organisms, and these were collectively named cephalosporin P [10]. The separation of these molecules led to the discovery of Cephalosporin N [10] (which was later renamed Penicillin N) which exhibited activity against both Gram positive and Gram negative organisms [11]. Also present was a small amount of a compound named Cephalosporin C [12]. This compound was found to have a similar spectrum of activity to penicillin N, but was found to be resistant to hydrolysis by penicillinase and was later shown to have a β -lactam ring, but a dihydrothiazine (cephem) ring replaced the thiazolidine ring of the penams [12] as shown in Figure 1.2 overleaf.

1.3.2. 7-Cephalosporanic Acid (7-ACA).

Figure 1.2: Structure of 7-Cephalosporanic Acid.



After 7-ACA had become available in quantity, many semi-synthetic derivatives were produced, some with similar acyl groups to benzylpenicillin and the semi-synthetic penicillins. This led to the production of cephalothin and cephaloridine. These early cephalosporins were soon to be followed by cephalexin, cefaclor and many others.

The basic cephalosporin nucleus consists of the essential β -lactam ring fused to a dihydrothiazine ring. Modifications of the cephem nucleus at position 7 by the addition of methoxy groups (forming cephamycins) increase β -lactamase stability but decrease activity against Gram positive species because of the lower affinity of these compounds for the penicillin binding proteins [13]. The more useful acyl side chains have been those that contain a 2-aminothiazoyl group, as this increases the affinity of the molecule for the penicillin binding proteins of Gram negative bacteria and streptococcal species. Iminomethoxy groups provide β -lactamase stability against the common plasmid β -lactamases from *Staph. aureus* and the TEM, SHV-1, OXA and PSE enzymes found in *Enterobacteriaceae* and *P. aeruginosa*, as well as the chromosomally mediated P99-type enzymes of *Enterobacter* spp. The newer cephalosporins have a much wider spectrum of activity compared with the first generation cephalosporins, although some

clinical bacteria have also become resistant to these newer compounds. Replacement of the sulphur atom in the cephalosporin dihydrothiazine ring by oxygen produces an increase in intrinsic activity in a number of cephalosporins [14].

The first cephalosporinase with the ability to hydrolyse cephalosporin C was reported in 1956 [15].

1.3.3. Classification of The Cephalosporins.

It was soon discovered that the substitution of the acyl side chains of 7-ACA could alter the antibacterial and pharmacokinetic properties of the compounds with this core structure. Hence, like the penicillins, many semi-synthetic compounds were produced with varying antibacterial spectra of activity.

Table 1.2: Classification System for Cephalosporins.

Cephalosporin Generation			
First	Second	Third	<i>Fourth</i>
Cefaclor	Cefamandole	Cefixime	Cefepime Cefpirome
Cefadroxil	Cefmetazole	Cefotaxime	
Cefazolin	Cefonicid	Cefoperazone	
Cephalexin	Ceforamide	Cefpodoxime	
Cephaloridine	Cefoxitin	Ceftazidime	
Cephalothin	Cefuroxime	Ceftizoxime	
Cephapirin		Ceftriaxone	
Cephradine			

A recent review of the classification of cephalosporins can be found in [16].

1.3.3.1. First Generation Cephalosporins.

The first generation cephalosporins include oral and parenteral compounds with moderate antibacterial activity, and are stable to staphylococcal β -lactamases. However, these compounds are susceptible to hydrolysis by a wide variety of enterobacterial β -lactamases.

1.3.3.2. Second Generation Cephalosporins.

These compounds are parenteral compounds with moderate antibacterial activity, and show resistance to a wide range of β -lactamases.

1.3.3.3. Third Generation Cephalosporins.

The third generation cephalosporins were heralded as the ultimate cephalosporins with stability against β -lactamase hydrolysis coupled with a wider spectrum of antibacterial activity. These compounds showed increased activity against Gram negative strains, and were designed to have increased anti-*Pseudomonas* activity [14]. These compounds were also designed to be resistant to hydrolysis by early TEM- and SHV- derived enzymes. However, whilst the TEM-1 and SHV-1 β -lactamases remained sensitive to these compounds, point mutations in the gene encoding these enzymes have occurred, resulting in an 'opening' of the active site to facilitate the binding and hydrolysis of these compounds by extended spectrum TEM- and SHV- derived enzymes such as TEM-3, TEM-5, and SHV-2.

1.3.3.4. Fourth Generation Cephalosporins.

Examples of this class of compounds include cefepime and ceftazidime. Structurally, these compounds resemble second generation cephalosporins in their substituted groups. However, ceftazidime is a zwitterionic 'bullet-shaped' molecule, engineered for rapid penetration into the cell. These compounds are resistant to hydrolysis by extended spectrum β -lactamases such as TEM-3 and TEM-5 but hydrolysed by all known metallo- β -lactamases with the exception of CphA [17].

1.4. Monobactams.

Naturally occurring compounds of this group also possess a β -lactam ring, but show limited antibacterial activity alone [18]. These compounds bind to penicillin binding proteins and it has been reported that sulbactam was efficacious in the treatment of both *Burkholderia cepacia* [19] and *Acinetobacter baumannii* [20].

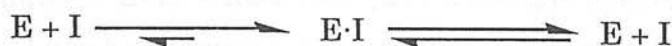
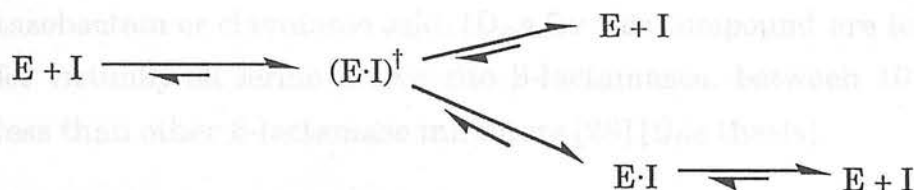
Monobactams are capable of inhibiting several β -lactamases by binding to and hence occupying the active site. Certain enzymes are capable of hydrolysing these compounds, although this hydrolysis is slow because the acyl-enzyme complex exists for a relatively long period of time before the hydrolysis product is released.

Semi-synthetic monobactams (6-desaminopenicillin sulphones) were developed, including aztreonam, sulbactam and tazobactam. Aztreonam was the first monobactam marketed, and was followed by sulbactam and tazobactam which exhibit a broader spectrum of activity and show greater stability than aztreonam.

1.5. β -lactamase Inhibitors.

When the molecular mechanism of drug resistance has been determined, it has been possible in some cases to design specific therapeutic combinations to overcome particular resistance determinants. If the resistance is mediated by an enzyme capable of inactivating the β -lactam agent, the formulation and hence co-administration of a β -lactamase inhibitor with a β -lactam agent will increase the half life of the antibacterially active β -lactam.

The mechanisms of β -lactamase inhibition mediated by these compounds can fall into any one of the several categories shown in Figure 1.3 overleaf.

Figure 1.3: Mechanisms of β -lactamase Inhibition.Competitive Inhibition.Non-Competitive "Time Dependant" Inhibition.Irreversible "Suicide" Inhibition.

E - Enzyme

I - Inhibitor

 P_x - Product $E \cdot I$ - Enzyme Inhibitor acyl intermediate

Adapted from [21].

The strategy of combining a β -lactam with a β -lactamase inhibitor [22], for example amoxycillin with clavulanic acid, is marketed as Augmentin¹ and remains active against many strains which produce a β -lactamase capable of hydrolysing amoxycillin. Clavulanic acid has a high affinity for class A β -lactamases such as PSE-4, SHV-1, and TEM-1 β -lactamases and is a potent inhibitor [23], but has poor antibacterial activity alone [24]. Clavulanic acid (an oxapenam) and sulbactam (a penicillin sulphone) have novel structures which resemble the nucleus of the penicillin molecule, and yet are not hydrolysed by the vast majority of these enzymes. These inhibitors work by either irreversibly binding to, and inhibiting the β -lactamase as a suicide inhibitor [25] or by potentiating the effect of the

¹ Trademark of SmithKline Beecham Pharmaceuticals.

β -lactam antibiotic. Sulbactam has been shown to inhibit β -lactamases in a similar way to clavulanic acid, although it is hydrolysed prior to inhibition [26]. Tazobactam, another monobactam is a slightly more effective inhibitor than sulbactam [21] although hydrolysis has been detected [27], and has been formulated with piperacillin and marketed as Tazocin².

BRL 42715, an experimental penem, is more effective against both chromosomal and plasmid mediated β -lactamases than sulbactam, tazobactam or clavulanic acid. ID_{50} s for this compound are less than 10nM for virtually all serine active site β -lactamases, between 10- and 100-fold less than other β -lactamase inhibitors [28] [this thesis].

1.6. The Carbapenems.

These compounds possess a β -lactam ring, as do all β -lactam compounds. However, compounds from this group possess an unsaturated penem ring, unlike the saturated penam ring of the penicillins and cephem ring of the cephalosporins. The olivanic acids are related to the carbapenems and were first isolated from a strain of *Strep. olivaceus*, but were so unstable that they have not been employed clinically [29].

The carbapenems have the broadest spectrum of all β -lactam antibiotics, encompassing both Gram positive and Gram negative organisms, aerobes, and anaerobes, fermenters and non-fermenters [30]. The carbapenems remain active against Gram negative strains that resist broad spectrum cephalosporins via stably de-repressed chromosomal Ambler class C β -lactamases, and also those strains which produce extended spectrum variants of TEM- and SHV- enzymes [29]. Potent carbapenemase activity was thought to be mediated solely by metallo- β -lactamases, although it has recently been reported in some serine active β -lactamases. Stability to common β -lactamases is not total, as imipenem hydrolysis has been detected in several Class 1 enzymes, but is usually very slow;

² Trademark of Lederle.

$V_{\max} = < 0.006\%$ V_{\max} of cephaloridine. It should be noted that imipenem is a strong inducer of β -lactamases, although the enzyme produced is usually unable to hydrolyse this compound [31].

1.6.1. Currently Available Carbapenems.

Imipenem is currently licensed for use in the UK and in many other countries around the world. It is marketed as Primaxin³, and is formulated with cilastatin, a renal dehydropeptidase 1 inhibitor with no antibacterial activity itself [32]. Imipenem (*N*-formamidoyl-thienamycin) is derived from thienamycin, a natural product of the soil organism *Streptomyces cattleya* [33], and it is the *N*-formamidoyl side chain which increased the chemical stability of thienamycin [34]. The stability of this compound in the presence of many β -lactamases is conferred by the *trans* configuration of the hydroxyethyl side chain on the β -lactam ring, which differs from the usual *cis* conformation of substitutions at this position [30].

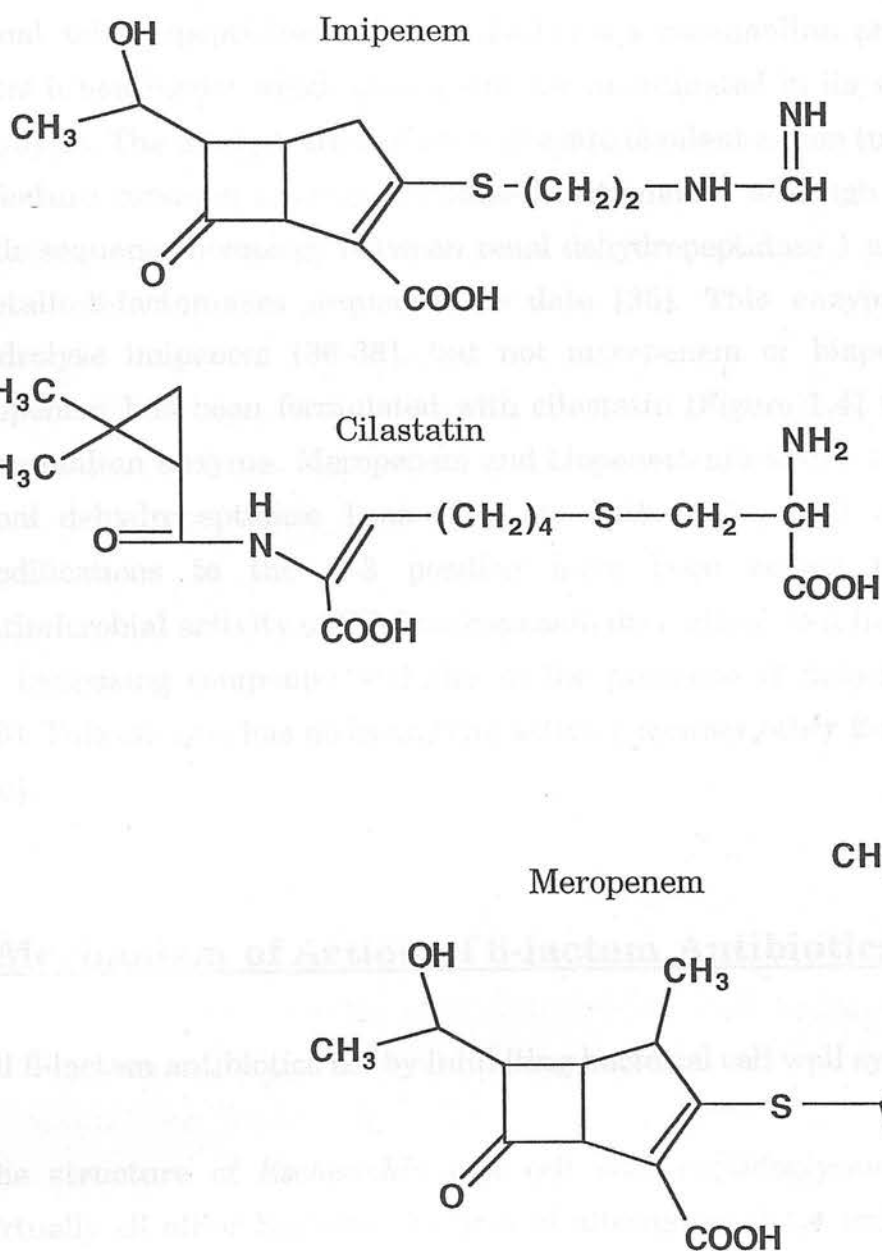
Meropenem⁴, another carbapenem, has also been licensed for use in Italy and Great Britain. The structure of these compounds are shown in Figure 1.4 overleaf together with the renal dehydropeptidase 1 inhibitor cilastatin which is co-administered with imipenem.

1.6.2. Mechanism of Action.

The mechanism of action of the carbapenem antibiotics is fundamentally the same as that for all other β -lactams, and is discussed in Section 1.7. Imipenem is one of the smallest β -lactam antibiotics. Its small size and zwitterion state enable it to gain access to the periplasmic space of Gram negative bacilli by passing through canals in the cell membrane created by porin proteins.

³ Trademark of Merck Sharp & Dohme.

⁴ Trademark of Zeneca Pharmaceuticals.

Figure 1.4: Structure of Currently Available Carbapenems.

Whilst cilastatin, the renal dehydropeptidase 1 inhibitor, is not a carbapenem, it has been included in the above figure because it is co-administered with imipenem. Meropenem requires no such inhibitor since it is stable in the presence of this mammalian enzyme.

1.6.3. Carbapenems and Renal Dehydropeptidase 1

Renal dehydropeptidase 1 (EC 3.4.13.11) is a mammalian protease of the renal brush border which uses a zinc ion co-ordinated in its active site for catalysis. The incorporation of an active site divalent cation (usually zinc) is a feature common amongst metallo- β -lactamases, although there is very little sequence homology between renal dehydropeptidase 1 and any of the metallo- β -lactamases sequenced to date [35]. This enzyme is able to hydrolyse imipenem [36-38], but not meropenem or biapenem, and so imipenem has been formulated with cilastatin [Figure 1.4] to inhibit this mammalian enzyme. Meropenem and biapenem are stable to mammalian renal dehydropeptidase 1 as they are both methylated at position 1. Modifications to the C-3 position have been shown to affect the antimicrobial activity of PS-5 carbapenem derivatives, but have little effect on increasing compound stability in the presence of dehydropeptidase 1 [39]. This enzyme has no hydrolytic activity against other β -lactam agents [40].

1.7. Mechanism of Action of β -lactam Antibiotics.

All β -lactam antibiotics act by inhibiting bacterial cell wall synthesis.

The structure of *Escherichia coli* cell wall peptidoglycan, and that of virtually all other bacteria consists of alternating β -1,4 linked residues of *N*-acetyl-D-glucosamine (NAG) and of *N*-acetylmuramic acid (NAM). The strict alternation of NAG and NAM in the glycan is ensured by the synthesis of disaccharide repeating units prior to polymerisation. These disaccharides are themselves linked β -1,4 so that the glycan becomes a modified form of chitin [41].

All peptidoglycans initially carry a pentapeptide subunit (L-Ala-D-Glu-mDAP-D-Ala-D-Ala) on each D-lactyl group of the NAM moiety. This is thought to be common to all Gram negative and some Gram positive

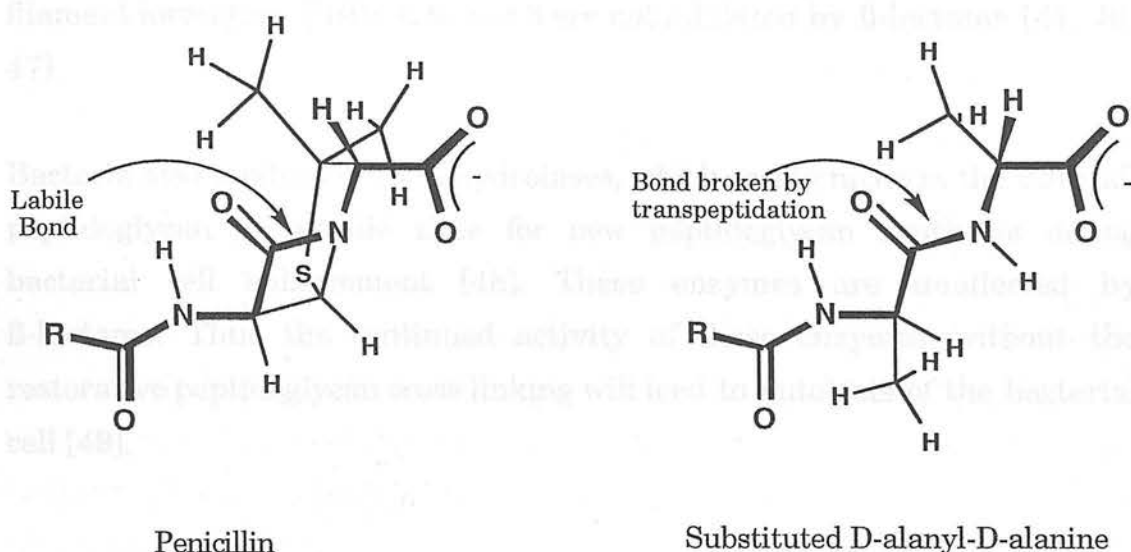
organisms. However, this may be further modified by transpeptidation or carboxypeptidation.

The final step of cell wall synthesis is the transpeptidase catalysed cross linking of the peptidoglycan chains. Cross linkage involves the elimination of a D-Ala residue from the N-terminal peptide unit, thus cleaving the D-alanyl-D-alanine linkage, followed by formation of a D-alanyl-acceptor cross link.

The mature peptidoglycan is thus crosslinked into a single polymer which forms a sheath around the bacterial cell. This peptidoglycan polymer layer is thought to be thinner in some Gram negative organisms than in Gram positive organisms, possibly a mono-layer, as Gram negative organisms also possess an outer membrane to protect the peptidoglycan from degradative attack. As this membrane is not present in Gram positive organisms, the peptidoglycan is thought to exist as a multi-layered structure to which other polymers are covalently linked.

Penicillin binding proteins are penicillin sensitive enzymes found in all bacteria which catalyse these terminal steps in the assembly of peptidoglycan, and can be D-D-carboxypeptidases, transglycosylases or transpeptidases. They are quantitatively a minor component of the plasma membrane and vary from species to species, with molecular masses between 30kDa and 150kDa, and are numbered in order of decreasing size [41, 42].

β -lactam antibiotics are structural analogues of acyl D-alanyl-D-alanine donor substrates for transpeptidation. This observation was first reported in 1965 [43], and is shown in Figure 1.5 overleaf. The β -lactam antibiotic will bind to the PBPs and thus inhibit peptidoglycan cross-linking leading to bacterial cell death.

Figure 1.5: Similarities of β -lactams and D-alanyl-D-alanine.

Adapted from [43].

A recent report examined the relative binding affinities of biapenem, imipenem, and meropenem to the various PBPs of *E. coli*, *P. aeruginosa* and *Staph. aureus* [44]. The main advantage of this study was that the same PBP preparations were used for all assays, and the same methodology was employed, allowing the resulting data to be compared directly. This study reinforced the previous report that the affinities of the various PBP for β -lactams vary from species to species [45].

All three carbapenems bound tightly to PBP-1 of *Staph. aureus*. In *E. coli*, all three carbapenems had high affinities for PBPs 2 and 4. However, meropenem bound to PBPs 2 and 4 from *P. aeruginosa* with greater affinity than biapenem or imipenem. In general, meropenem was reported to have the broadest PBP profile in Gram negative organisms, although biapenem showed the best PBP profile against *Staph. aureus*.

In *E. coli*, β -lactams which bind to PBPs in group 1 are the most potent at effecting cell lysis. PBPs 1_A and 1_B are involved in cell elongation. PBP-2 is thought to mediate a transpeptidase or carboxylase reaction as β -lactams

which preferentially bound to PBP-2 caused ovoid cell morphology. PBP-3 is involved in cell division, and β -lactams which bound to this PBP caused filament formation. PBPs 4, 5, and 6 are not inhibited by β -lactams [41, 46, 47].

Bacteria also produce murein hydrolases, which create nicks in the cell wall peptidoglycan to provide sites for new peptidoglycan synthesis during bacterial cell enlargement [48]. These enzymes are unaffected by β -lactams. Thus the continued activity of these enzymes without the restorative peptidoglycan cross linking will lead to autolysis of the bacterial cell [49].

1.8. Resistance to β -lactam Antibiotics.

Resistance to β -lactam antibiotics is mediated through either a minor or major DNA rearrangement.

An example of a minor rearrangement is a point mutation. Such a mutation could alter the level of expression of a gene giving the organism a selective advantage over others. A point mutation could alter the sequence of a translated protein. If this protein were the target of the antimicrobial, the mutation could alter the target protein such that the antibacterial agent could no longer bind to its target. If this protein were an enzyme capable of destroying the antimicrobial, such a mutation could alter its specificity or spectrum of activity [50-53].

There are several examples of major rearrangements, including gene duplication, and the acquisition of foreign DNA. The acquisition of foreign DNA may include acquisition of resistance determinants, and such transfers can be mediated by plasmid conjugation, as in the case of TEM-1 [54]. Bacteriophages, transposons and integrons have also been shown to have the ability to carry resistance determinants [55-58]. The ability of bacteria to acquire and disseminate genetic material is well illustrated by

the dramatic increase in the frequency with which resistant organisms have been isolated from clinical infections.

There are several mechanisms by which bacteria may become resistant to β -lactam antibiotics.

1.8.1. Permeability.

β -lactam resistance has been linked to a reduction of membrane permeability [59, 60], a resistance mechanism relying on the exclusion of the β -lactam compound from the target site. Imipenem resistance mediated by this mechanism was first reported in *P. aeruginosa* in 1986 [61] and has also been observed in several other species. In *P. aeruginosa*, it has been linked to the loss of porin protein D2 [62] and F [59]. Porin D2 has been shown to facilitate the penetration of imipenem across the outer membrane [63] and maps to between 71 and 75 minutes on the PAO1 chromosome [64]. In 1991, Kapotas and co-workers [65] acclimatised a strain of *P. aeruginosa* to imipenem and observed the concomitant loss of 6 membrane proteins, namely porins D1, D2, E, protein H1, lipoprotein H2 and an unidentified 85kDa protein, although this strain remained sensitive to azlocillin and polymyxin B. Recently the efficacy of the new carbapenem BMS-181139 has been shown to be independent of porin D2 levels, unlike imipenem, meropenem and biapenem [66], and is the first report of such independence on porin levels. This porin independent penetration is thought to be more rapid than the D2 dependent pathway and has been attributed to the presence of a basic substituted group on position 1 or 6 of the penem nucleus [67].

Whilst the loss of outer membrane porins in Gram negative bacteria has been shown to lead to decreased susceptibility to a variety of antibiotics, it has been possible to modify β -lactams to take advantage of facilitated entry mechanisms into the periplasm by means of solute specific scavenging systems [68-70]. See [71] for a recent review.

1.8.2. Change In Target Site - Penicillin Binding Proteins (PBPs).

Resistance to β -lactam compounds can also be mediated by alteration of the target site. This was characterised in a *P. aeruginosa* clinical isolate as an alteration in PBP-4 which occurred during imipenem therapy, although no concomitant loss of membrane porin proteins was observed in this strain. This alteration led to a 16-fold increase in imipenem MIC, but MICs of all other antimicrobials remained unchanged [72]. In other Gram negative bacilli, penems have been shown to bind to PBP-2 [44,73] whereas other β -lactams bind to primarily to PBP-1_A, PBP-1_B and PBP-3 [44,74].

Organisms may become resistant to β -lactams by either altering existing PBPs so that they no longer bind penicillins, or the bacteria may acquire supplementary resistant PBPs. An example of this is a report of a *Staph. aureus* strain in which PBP-2 and PBP-3 were lost and replaced with PBP-2' [75]. PBP-2' functionally complemented PBP-2 and PBP-3, and yet was not susceptible to many β -lactams.

1.8.3. Permeability and Hyper-production of a β -lactamase.

The combination of a reduction in outer membrane permeability and the production of a β -lactamase have been shown to cause resistance to many β -lactam antibiotics, including imipenem [76] even though the β -lactamase produced may be unable to hydrolyse carbapenems at a significant rate. The permeability barrier reduces the concentration of the β -lactam agent in the periplasm such that the low level β -lactam hydrolysis by the enzyme is sufficient to protect the organism from this agent. This has been well documented in *P. aeruginosa* isolates, and Enterobacteria which hyper-produced a P99 type β -lactamase.

1.8.4. Efflux Mechanism.

Several membrane associated energy driven efflux mechanisms with broad substrate specificity have been described, mainly in *P. aeruginosa*. These efflux systems confer resistance to antimicrobials from many different classes, including tetracycline, chloramphenicol and fluoroquinolones [77, 78]. It has also been shown that such an efflux mechanism can increase the resistance of isolates to β -lactams [79].

1.8.5. Enzymatic Degradation of β -lactam.

The last, and most important form of resistance is mediated by the hydrolysis, and hence inactivation of the β -lactam ring of the compound. This is caused by the action of a β -lactamase enzyme. β -lactamases (EC 3.5.2.6) form a large family of enzymes that can hydrolyse the β -lactam ring of antimicrobial agents which possess this bio-active structure, but do not interfere with peptidoglycan metabolism as they lack DD-peptidase activity. Ironically, the first report of a β -lactamase [80] was published in the same year that penicillin underwent its first clinical trial in humans. Since then, the use of β -lactams has led to an increase in the prevalence of β -lactam resistance amongst bacteria. In 1940, virtually all strains of *Staph. aureus* world-wide were sensitive to penicillin. However, just over ten years after its first clinical use, 73% of *Staph. aureus* isolates from in-patients at the Boston City Hospital were resistant to penicillin, and most of these organisms produced a β -lactamase [81]. By 1975, 84% of the in-patient *Staph. aureus* isolates at the Massachusetts General Hospital were resistant to penicillin [82], and by 1986 this figure had increased to more than 90% of *Staph. aureus* being penicillin resistant [83].

Resistance to β -lactam antibiotics can be mediated by the production of a β -lactamase, or by other mechanisms, but these are not mutually exclusive [84]. The production of a β -lactamase is the most common mechanism of resistance to this class of antibacterial compounds. These enzymes are discussed in greater detail in Section 1.9 overleaf.

1.9. β -lactamases.

Over the last 30 years, many different β -lactamases have been described from both Gram positive and Gram negative bacteria. There are currently 196 different β -lactamases known to exist [85]. The vast majority of these enzymes use a serine residue in the active site to hydrolyse the β -lactam ring and these enzymes vary considerably in their ability to hydrolyse many different β -lactam antibiotics. The other type of β -lactamase contains a metal ion within its active site.

1.9.1. Origin of β -lactamases.

Kelly and co-workers [86] have proposed that β -lactamases evolved from penicillin sensitive DD-peptidases by comparing the structure of DD-peptidases with the β -lactamase from *B. licheniformis* 749/C. Whilst these proteins lacked primary sequence homology, they shared a similar 3-dimensional structure, and some residues were conserved around the active site [86, 87]. The evolution of Class A β -lactamases has also been examined [88]. Kirby [89] investigated this further by constructing a phylogenetic tree and postulated that Class A and Class C β -lactamases evolved from an actinomycete β -lactamase, which itself had evolved from DD-peptidases. See Section 1.9.2 for β -lactamase classification.

There have not been any reports suggesting an evolutionary origin for Class B β -lactamases, but since these enzymes share neither sequence homology or mechanism of action with serine active site β -lactamases, it may be possible that parallels could be drawn with other enzymes which incorporate a zinc ion as part of the active site [90] including cytidine deaminases, angiotensin converting enzymes and matrix metallo-proteinases. See [91] for a recent review.

1.9.2. Classification of β -lactamases.

Several schemes have been devised to classify β -lactamases. The first was described by Richmond and Sykes [92] and was based upon substrate affinities, iso-electric points and inhibition data. Since this classification system was introduced, many more β -lactamases have been described, and other classification systems based upon new criteria have been devised which include these newer enzymes.

The Ambler system [93] was proposed in 1980, and remains the most widely used classification system today. It is based on amino acid sequence homology around the active sites of the enzymes, and a standard numbering system for the amino acids in the sequence has also been proposed [94]. Since its publication, other classes have been added to this classification scheme, and these classes are discussed later.

The Bush classification scheme [95, 96] was developed in response to the plethora of enzymes that were discovered with different properties. This scheme is based upon the Richmond and Sykes classification, but has been modified to include both physical and kinetic data. A recent report updated the Bush classification scheme [85] with the addition of new groups to include novel β -lactamases which had been characterised since the publication of the original scheme. However, the Ambler [93] system is the most widely accepted. Table 1.3 overleaf compares these classification systems.

Table 1.3: Comparison of β -lactamase Classification Schemes.

1980 Ambler Class [93]	1995 Bush Jacoby Madeiros [85]	1989 Bush group [95, 96]	1973 Richmond-Sykes [92]	Preferred substrates	Inhibited by		Representative enzymes
					Clav	EDTA	
A	2a	2a	Not included	Pen	+	-	Penicillinases from Gram + bacteria
	2b	2b	III	Pen / NS Ceph	+	-	TEM-1, TEM-2, SHV-1
	2be	2b'	Not included [¶]	Pen / BS Ceph	+	-	TEM-3 to TEM-26 SHV-2 to SHV-6
	2br	Not included	Not included	Pen	+/-	-	TEM-30 to TEM-36, TRC-1
	2c	2c	II, V	Pen / Carb	+	-	PSE-1 PSE-3 PSE-4
	2e	2e	Ic	Ceph	+	-	L2, MEN-1
	2f	Not included	Not included	Pen / Ceph / Carb	+	-	Nmc-A, Sme-1
B	3	3	Not included	Pen / Ceph / Carb	-	+	IMP-1, <i>B. cereus</i> II, Cfa, ESP
C [‡]	1	1	Ia, Ib, Id	Ceph	-	-	AmpC type β -lactamases, MIR-1
D [§]	2d (4)	2d	V	Pen / Clox	+/-	-	OXA-1 to OXA-11, PSE-2
E [†]	(3)	(3)	Not included	Pen / Ceph / Carb	-	+	L1

[¶] except K1 from Richmond-Sykes class IV

[‡] Ambler class C proposed by Jaurin and Grundstrom [97].

[§] Ambler class D proposed by Huovinen and co-workers [98].

[†] Ambler Class E proposed by Sanders [99].

Pen - penicillins

NS Ceph - narrow spectrum cephalosporin

Carb - carbapenems

Cloxacillin.

BS Ceph - broad spectrum cephalosporin.

Adapted from [85].

It can be seen from Table 1.3 that the Ambler system classified the enzymes into a smaller number of classes than the Bush system. The Ambler classification system will be used throughout this thesis unless otherwise stated.

The formation of an extra class to accommodate the homo-tetrameric zinc metallo- β -lactamase from *Sten. maltophilia*, has been proposed by Sanders [99], although the need for this additional class remains to be proven as this is the only enzyme currently in this class.

1.9.2.1. Ambler Class A.

All β -lactamases within this class catalyse β -lactam hydrolysis via a serine residue usually found at position 70 within the active site. These enzymes vary considerably in their ability to hydrolyse many different β -lactam antibiotics, ranging from strict penicillinases to broad spectrum β -lactamases and carbenicillinases. This class of enzymes includes all TEM-derived and SHV-derived enzymes which are the most prevalent β -lactamases found in Gram negative isolates.

Enzymes of this class may be chromosomally or plasmid encoded and are, with the exception of TRC-1 [100], generally regarded as sensitive to clavulanic acid, tazobactam and other β -lactamase inhibitors.

In the last two years, several class A enzymes from this class have been characterised which have the ability to hydrolyse carbapenems [101, 102 - see Table 1.5], a compound previously thought to be stable to all Class A enzymes. However, the carbapenem hydrolysing Class A enzymes do still show some sensitivity to β -lactamase inhibitors, including clavulanic acid and tazobactam.

1.9.2.2. Ambler Classes B and E.

Enzymes of these classes require a divalent cation, usually zinc for activity. These metallo- β -lactamases are able to hydrolyse penicillins, cephalosporins and carbapenems, although penicillins tend to be their preferred substrate. For this reason, they have been referred to as metallo-penicillinases in some publications. Metallo- β -lactamases are not susceptible to any β -lactamase inhibitors in clinical use, such as clavulanic acid or sulbactam, which exert their effect by inactivating active site serine residues. In fact sulbactam has been shown to be hydrolysed by these enzymes [103], as has the experimental penem BRL 42715. Despite being a substrate for enzymes of this class, BRL 42715 is the only known β -lactam with the ability to inhibit these enzymes at physiologically attainable concentrations [this thesis], most probably by acting as a competitive substrate for the metallo- β -lactamase.

An overview of the kinetic parameters of enzymes from this class can be found in [103] and [17].

These enzymes are generally monomeric proteins with molecular masses of 25-35 kDa, similar to the serine active site enzymes. They are readily inhibited by chelating agents such as EDTA, dipicolinic acid, and *o*-phenanthroline which are thought to inactivate the enzyme by sequestering the metal cation from the active site. The only currently known exceptions to this are the L1 enzyme from *Sten. maltophilia*, which is thought to exist as a homo-tetramer with an overall mass of approximately 118kDa and, in 1990 an imipenem hydrolysing β -lactamase with an estimated molecular mass of over 60kDa was reported from *Bacteroides distasonis* but this enzyme was insensitive to EDTA, and so may not belong to this class of enzymes.

These enzymes are usually chromosomally encoded. The only transferable metallo- β -lactamases have been reported from *P. aeruginosa* [104], *Bact. fragilis* [105] and *S. marcescens* [106]. In the *P. aeruginosa* and *Bact. fragilis* isolates, the metallo- β -lactamase gene was coded on a plasmid. These

plasmids were transferable to other strains of the same species with a concomitant transfer of imipenem resistance, although attempts to transfer imipenem resistance to *E. coli* were unsuccessful, and no plasmid transfer was detected.

1.9.2.3. Ambler Class C.

The formation of this class was proposed by Jaurin and Grundstrom who, having sequenced the *ampC* gene encoding a cephalosporinase from *Escherichia coli* K-12, found little sequence homology with Class A penicillinases [97]. Class C enzymes also utilise a serine residue within the active site like class A enzymes, but overall sequence homology between enzymes of these two classes is low. These enzymes are generally chromosomally encoded inducible cephalosporinases and are generally regarded as resistant to inhibition by clavulanic acid [107] and other β -lactamase inhibitors. Examples of enzymes belonging to this class include P99 and AmpC.

The kinetic parameters of six enzymes from this class have been determined and compared [108].

1.9.2.4. Ambler Class D.

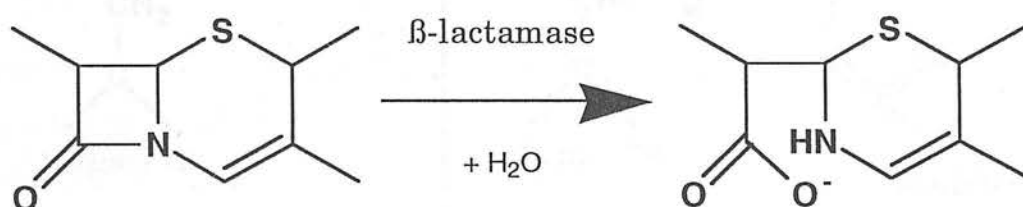
Enzymes of this class are serine active site enzymes and have been found in both Gram positive and Gram negative organisms. They are sensitive to clavulanic acid. This class was proposed by Huovinen and co-workers who sequenced PSE-2 [98]. This class also includes all OXA- derived enzymes.

Ledent and co-workers have studied and compared the kinetic parameters of three β -lactamases from this class [109].

1.9.3. Mechanism of Action of β -lactamases.

The basic principle of β -lactam hydrolysis is similar for both metallo- and serine active enzymes, namely the hydrolysis of the β -lactam ring to form an acid product with no antibacterial activity as shown in Figure 1.6 below. However, the mechanism of hydrolysis varies between these two types of enzymes.

Figure 1.6: Schematic Mechanism of β -lactam Hydrolysis.



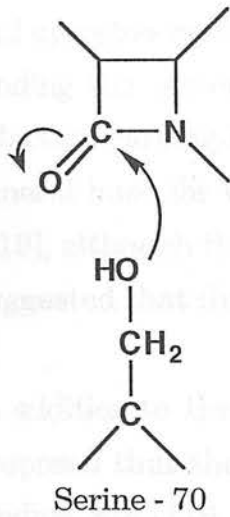
1.9.3.1. Serine Active β -lactamases.

β -lactamases of Ambler classes A and C and D [see Section 1.9.2] all possess a serine residue at position 70 (Ambler numbering system as proposed in [94]). This residue is essential for catalytic activity and mutation of this residue results in almost, if not total, inactivation of the enzyme [110]. A Lys-Thr(Ser)-Gly triad is also conserved around the active site [111]. Serine-70 is thought to act as a nucleophile, attacking the carbonyl group in the β -lactam ring to which it becomes covalently bound forming an acyl-enzyme intermediate. Lysine 73 is thought to act as a general base, transferring a proton from serine-70 to the nitrogen atom in the β -lactam ring via serine-130 [112].

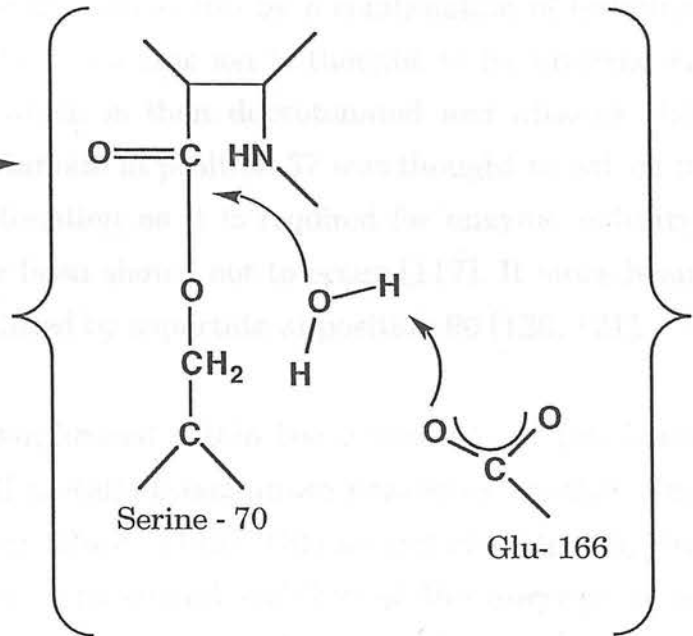
Site directed mutagenesis of β -lactamase I from *Bacillus cereus* 569/H and other enzymes have shown that Glu-166 may activate the catalytic water (designated water-673) molecule for deacylation of the enzyme intermediate by causing fission of the bond between the enzyme and inactive β -lactam [112-114]. Figure 1.7 overleaf describes this hydrolytic mechanism.

Figure 1.7: General Mechanism of Serine-Active β -lactamases.

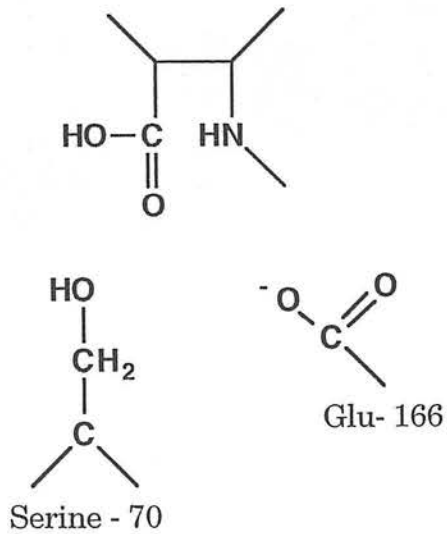
Enzyme Substrate Complex



Acyl-Enzyme Intermediate



Enzyme Product Complex

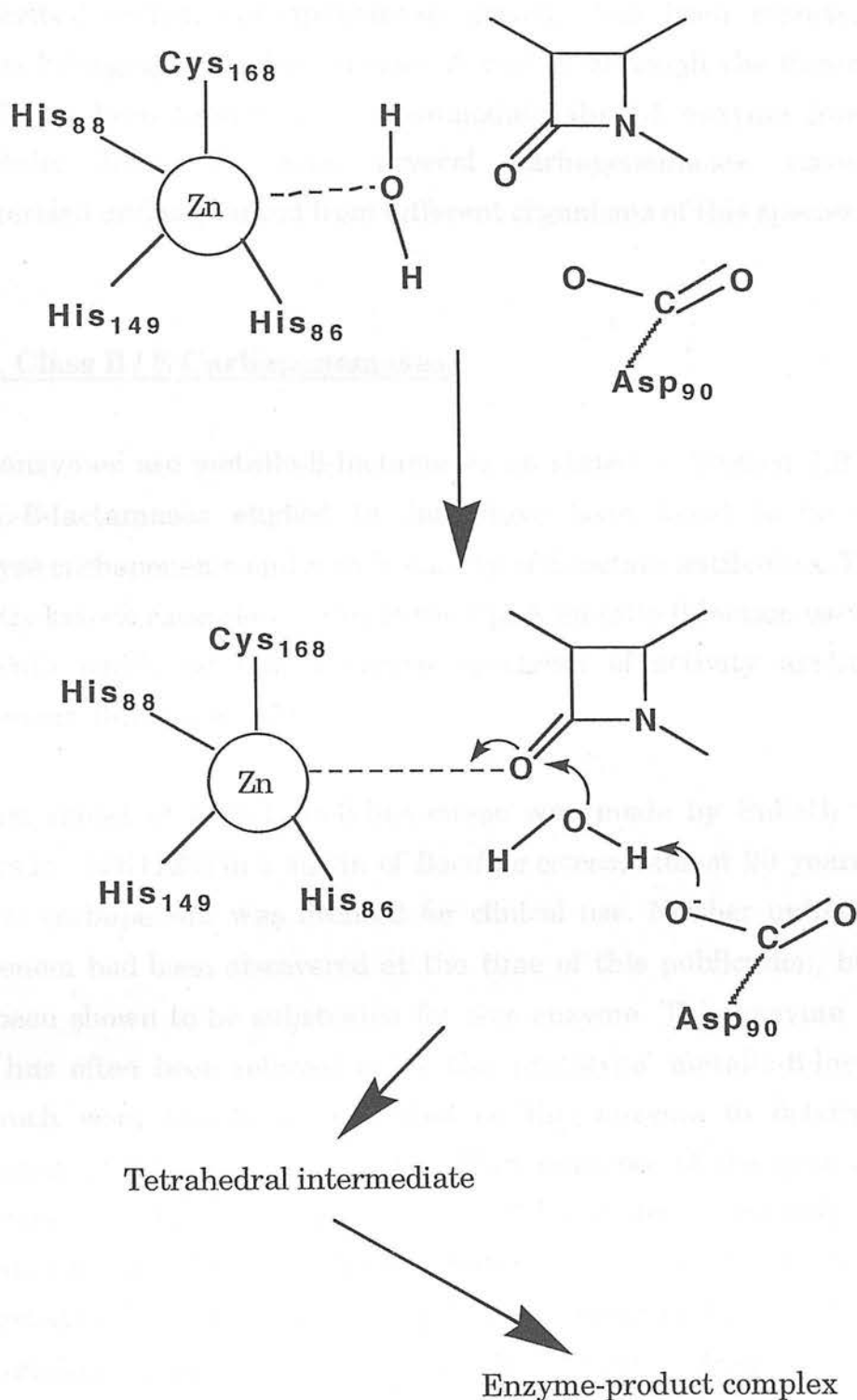


Adapted from [40, 112, 113, 115].

1.9.3.2. Metallo- β -lactamases.

Enzymes of this class require a divalent cation, usually zinc for activity. This ion is co-ordinated within the active site by a combination of histidine and cysteine residues [116-118]. The zinc ion is thought to be involved in binding the water molecule which is then deprotonated and attacks the β -lactam carbonyl group. Glutamate at position 37 was thought to act as a general base for water deprotonation as it is required for enzyme activity [119], although this has since been shown not to occur [117]. It has since been suggested that this role is fulfilled by aspartate at position 90 [120, 121].

In addition to the zinc ion co-ordinated within the active site, it has been proposed that the *B. cereus* II metallo- β -lactamase possesses another zinc binding site [116, 122] of lower affinity [118]. This second zinc ion may be responsible for maintaining conformational stability of the enzyme. It is unclear at present whether other metallo- β -lactamases also possess more than one zinc binding site. Figure 1.8 overleaf describes the proposed mechanism for the metallo- β -lactamase hydrolysis of a β -lactam.

Figure 1.8: General Mechanism of Metallo- β -lactamases.

Adapted from [119].

1.9.4. Carbapenemases.

As described earlier, carbapenemase activity has been reported from enzymes belonging to Ambler classes A and B, although the formation of class E has been proposed to accommodate the L1 enzyme from *Sten. maltophilia* [99]. To date, several carbapenemases have been characterised and sequenced from different organisms of this species.

1.9.4.1. Class B / E Carbapenemases.

These enzymes are metallo- β -lactamases as stated in Section 1.9.2.2. All metallo- β -lactamases studied to date have been found to be able to hydrolyse carbapenems and a wide variety of β -lactam antibiotics. The only currently known exception to this is the CphA metallo- β -lactamase from *A. hydrophila* which exhibits a narrow spectrum of activity against non-carbapenem β -lactams [17].

The first report of a metallo- β -lactamase was made by Sabath and co-workers in 1966 [123] in a strain of *Bacillus cereus*, almost 20 years before the first carbapenem was licensed for clinical use. Neither imipenem nor meropenem had been discovered at the time of this publication, but have since been shown to be substrates for this enzyme. This enzyme from *B. cereus* has often been referred to as the 'prototype' metallo- β -lactamase, and much work has been conducted on this enzyme to determine its mechanism of action [124, 125]. The DNA sequence of the gene encoding this protein has been determined [121, 133] and this is the only metallo- β -lactamase for which an X-ray crystallographic structure has been determined [117]. Table 1.4 overleaf lists all known or suspected metallo- β -lactamases characterised to date. The enzyme from a strain of *Alcaligenes faecalis* is also listed, but in a greyed box as this enzyme may be a metallo- β -lactamase as it shows a substrate and inhibitor profile consistent with known metallo- β -lactamases, but imipenem hydrolysis was not determined when this enzyme was characterised [126].

Table 1.4: Properties of Metallo- β -lactamases Characterised to Date.

Species	Strain	Enzyme	pI	Sequence Available?	EDTA Inhibition	Molecular Mass	% Homology with <i>Bacillus cereus</i> II	Reference
<i>Aeromonas hydrophila</i>	AE036	CphA [†]	8.0	Yes	Yes	28000 ^P	24.7	[127]
	AER19	A2h [†]	8.0		Yes	31500 ^P		[128]
	AER64	-	8.0		ND			[129]
	AER84	-	8.0		ND			[129]
	-	-	7.0-7.4		ND			[130]
<i>Aeromonas salmonicida</i>	ASA111	ASA1	>7.9		Yes	19000 ^P		[131]
<i>Aeromonas sobria</i>	AER14	A2s [†]	8.4		Yes	35000 ^P		[128]
	AER83	-	6.9 & 8.0		ND			[129]
	AER178	-	8.0		ND			[129]
	163a	-	9.3		Yes			[132]
<i>Aeromonas veronii</i>	ATCC 35624	-	8.0					[129]
<i>Bacillus cereus</i>	569H [†]	II	8.45	Yes [133]	Yes	24932 ^s (22500 ^P)	100	[134]
	5/B/6	II/5/B/6	8.4 / 9.1	Yes [121]	Yes	22000 ^P	90 ~90	[135] [135]
	10-73	-			Yes			[105]
<i>Bacteroides fragilis</i>	119		4.6		ND			[136]
	GAI-30079 [*]	-			Yes			[137]
	GAI-30144	-	4.7		Yes	33000 ^P		[138]
	G-237	-	4.8		ND	26000 ^P		[139]
	KSB-1468/90	-	4.5		Yes	31000 ^P		[140]
	QMCN3	CcrA type	~5.0	Yes	Yes	~25249	~32	[141]
	QMCN4	CcrA type	~5.0	Yes	Yes	~25249	~32	[141]
	TAL2480	CfIA	4.8	Yes	Yes	25249 ^s	32.5	[142] §
	TAL3636	CcrA	5.2	Yes [143]	Yes	26000 ^P	32	[216] §

Table 1.4. (Continued).

<i>Burkholderia cepacia</i>	-	PCM-1	8.45		Yes		[146]
<i>Flavobacterium odoratum</i>	GN14053	-	5.8		Yes	26000 ^p	[144]
<i>Legionella gormanii</i>	ATCC 33297	-	10.5		Yes	25000 ^p	[145]
<i>Pseudomonas aeruginosa</i>	GN17203	ESP	9.0		Yes	28000 ^p	[104]
<i>Serratia marcescens</i>	TN9106	IMP-1	>9.5	Yes	Yes	30000 ^p	[147]
	FHSM4055		8.7		Yes	30000 ^p	[148]
	AK9373	IMP-1 type					[106]
	AK9374	IMP-1 type					[106]
<i>Stenotrophomonas maltophilia</i>	AK9385	IMP-1 type					[106]
	GN12873	L1 ^o	6.9	No [149] [‡]	Yes	118000 ^g (26000) ^p	[151]
<i>Alcaligenes faecalis</i>	IID 1275	L1 ^o	6.9	Yes [150]	Yes	123000 ^g (30800) ^p	[124]
	GN14061	-	5.9		Yes	29000 ^p	[126]

† CphA and A2h/A2s may be identical. See Section 1.9.6.2.

‡ *Bacillus cereus* 569/H/9 not included as it is almost identical to 569/H.

§ This enzyme was found to have a substrate profile almost identical to GAI30144, and was not characterised further [138].

§ These enzymes first reported in 1986 [152] though little biochemical or sequence data known until 1990.

ND - Not determined.

CfIA and CcrA; the metallo- β -lactamases sequenced from *Bacteroides fragilis* have been shown to differ by only 2 amino acids. Substrate and inhibitor profiles seem unaffected by these substitutions [153].

g Molecular mass determined by gel filtration.

p Molecular mass determined by SDS-PAGE.

s Molecular mass calculated from DNA sequence.

§ Since the reports of these enzymes, it has been suggested that the L1 enzyme is heterogeneous between isolates [154, 155].

‡ This enzyme has been cloned and expressed in *Escherichia coli*, but not sequenced.

Alcaligenes faecalis enzyme has been listed in greyed box as this enzyme has similar biochemical and inhibitor properties to known metallo- β -lactamases, although imipenem hydrolysis of this enzyme was not determined [126].

1.9.4.2. Class A Carbapenemases.

It was originally thought that metallo- β -lactamases were the only β -lactamases with the ability to hydrolyse carbapenems, and that these compounds were stable in the presence of serine active site enzymes. The first serine active carbapenemase was discovered in an isolate of *S. marcescens*, and was reported by Yang and co-workers [156] although this enzyme was originally thought to be a metallo- β -lactamase until its sequence was determined in 1994 [102]. Also in 1990, a report was made characterising a β -lactamase from *Bacteroides distasonis* with the ability to hydrolyse imipenem, but this enzyme appeared resistant to inhibition by EDTA and thus may be a class A enzyme [157]. The molecular mass of this enzyme (>60000) was inconsistent with β -lactamases of this class and also with β -lactamases of class B. In 1993, Nordmann and co-workers correctly reported a serine active carbapenemase designated NmCA from an isolate of *Enterobacter cloacae* [101], an enzyme whose sequence has since been determined [158].

Table 1.5 overleaf compares the properties of all serine active site β -lactamases with carbapenemase activity which have been examined to date.

Table 1.5: Properties of Serine Active Site Carbenemases Characterised to Date.

Species	Strain	Enzyme	pI	Sequence Available?	EDTA Inhibition	Molecular Mass	Inhibitor Sensitivity	Reference
<i>Acinetobacter baumannii</i>	6B92	ARI-1	6.65		No	23000 ^g	Clav- R	[159]
<i>Bacteroides distasonis</i>	TAL7860	-	6.9		No	>60000 ^g	Clav- S	[157]
<i>Enterobacter cloacae</i>	NOR-1 1413B	NmcA	6.8	Yes [158]	No	29100 ^s	Clav- S	[101]
		Imi-1	7.1		No		Clav- S	[160]
<i>Serratia marcescens</i>	S6	Sme-1	9.7	Yes [102]	Yes	29300 ^s	Clav- S	[156]

Clav-R Resistant to inhibition by clavulanic acid.
Clav-S Sensitive to inhibition by clavulanic acid.
g Molecular mass determined by gel filtration.
p Molecular mass determined by SDS-PAGE.
s Molecular mass calculated from DNA sequence.

It should be noted that enzyme inhibition by chelators is not always conclusive evidence of a metallo-enzyme as several class A carbapenemases have been shown to be sensitive to EDTA inhibition despite having no metal ion co-ordinated at the active site [102, 156].

1.9.5. Sequence Homology Between Metallo- β -lactamases.

As yet there have been few reports of metallo- β -lactamases which have included sequence determination. As a result, there are limited data currently available on the heterogeneity of metallo- β -lactamase DNA sequences within each species. The DNA sequence of metallo- β -lactamases from several strains of *B. cereus* have been determined, although these strains were related. The only other genus for which the sequence of several metallo- β -lactamases have been determined, is *Bacteroides*. The enzymes of this genus show very high sequence homology and only differ in two amino acids [153], however the sequence of the only plasmid mediated metallo- β -lactamase from this species has not yet been determined [105]. The total sequence homology between metallo- β -lactamases of different species varies between 21% and 39% when compared with *B. cereus* II. Five distinct regions of homology have been identified in these metallo-enzymes [127], with deduced sequence homology of these enzymes increasing up to 80% around the active site, with almost all of these enzymes possessing three conserved histidines and a cysteine residue for metal ion co-ordination.

Table 1.6 overleaf describes the relative homology of metallo- β -lactamases sequenced to date. It should be noted however, that regions at the far C- and N-terminus were eliminated for normalisation purposes prior to alignment [103].

Table 1.6: To Show % Identity of Metallo- β -lactamases Sequenced to Date.

Enzyme	% Identity		
	<i>Bact. fragilis</i> CfA	<i>B. cereus</i> II 5/B/6	<i>A. hydrophila</i> CphA
<i>A. hydrophila</i> CphA	34.3	34.9	-
<i>B. cereus</i> II 5/B/6	41.4	-	-
<i>Sten. maltophilia</i> L1	22.7	21.4	21.0
<i>S. marcescens</i> IMP-1	35.9	38.9	22.1

Adapted from [103].

No sequence homology has been detected between metallo- β -lactamases and serine active carbapenemases. However, the serine active site carbapenemases Nmc-A from *Enterobacter cloacae* NOR-1 and Sme-1 from *Serratia marcescens* S6 share 70% amino acid identity [102]. The most similar non-carbapenem hydrolysing enzyme was the extended spectrum β -lactamase MEN-1 from *Escherichia coli*, which shared 50% amino acid identity with Nmc-A [158], although the homology of these carbapenemases with other class A β -lactamases was low.

1.9.6. Clinical Relevance of Carbapenemase Expression.

Whilst resistance to carbapenem antibiotics is rare, some isolates have been shown to be resistant to virtually all β -lactams and also to antimicrobials of other classes. Examples of these organisms include *Acinetobacter*, *Pseudomonas*, *Stenotrophomonas* isolates and *Burkholderria* strains from cystic fibrosis (CF) patients.

The production of a carbapenem hydrolysing β -lactamase is currently thought to represent the main threat to the use of carbapenems for serious nosocomial infections, although it should also be noted that neither metallo- β -lactamases nor serine active carbapenemases are the sole mechanism of resistance to carbapenems as described earlier in Section 1.8.

1.9.6.1. *Acinetobacter* spp.

Organisms from the genus *Acinetobacter* are opportunistic nosocomial pathogens which are encountered predominantly in ITUs and have been implicated in pneumonia, meningitis, endocarditis and septicaemia. To date, only one carbapenem hydrolysing β -lactamase has been characterised from this species [159]. This enzyme appeared to be a serine active site enzyme, and has recently been shown to be transferable to another *Acinetobacter* strain [Scraife, Young, Paton and Amyes, Personal Communication].

1.9.6.2. *Aeromonas* spp.

These organisms are widely distributed in the environment, and are increasingly being recognised as human pathogens. They have been implicated in a wide range of enteric diseases including gastro-enteritis, soft tissue infections and bacteraemia. These organisms can be found both in marine and fresh water environments, and there have been many reports of the presence of these bacteria in raw meats and fish, suggesting that this may be a food-borne pathogen. *Aeromonas hydrophila* is the species from this genus most frequently isolated in clinical specimens [161]. Clinical infections involving this organism are usually polymicrobial. The β -lactamases produced by this genus found to date have been chromosomally encoded and inducible.

The *Aeromonas* CphA enzyme has been shown to have a narrow substrate profile, with low hydrolysis rates of penicillins and cephalosporins including the chromogenic cephalosporin, nitrocephin [162]. It has also been reported that the carbapenemase activity of one *Aeromonas salmonicida* strain was caused by a β -lactamase which was unable to hydrolyse nitrocephin [131]. These reports contradict the observation by Iaconis and Sanders who stated that the A2 enzyme hydrolysed carbapenems, penicillins and nitrocephin [128]. This substrate profile was probably caused by the co-purification of the CphA-type carbapenemase with a cephalosporinase which both have a similar pI. This may explain the observation by this group that the apparent sensitivity of the A2 enzyme to the inhibitor clavulanic acid appeared to be substrate dependant [128].

In a recent study of 114 *Aeromonas* isolates [163], all *A. hydrophila* and *Aeromonas veronii* strains positively hybridised with a gene probe containing the *CphA* gene and its flanking regions. 42% of all the *Aeromonas* strains in the study hybridised with the probe, and carbapenemase activity was detected in 83% of hybridisation positive isolates. These data suggest that all *A. hydrophila* and *A. veronii* strains, the two most clinically relevant species of this genus, may encode a metallo- β -lactamase. Despite this, the MIC₉₀ of imipenem for all these strains was less than the NCCLS breakpoint at an inoculum of 10⁶ per ml, indicating that carbapenems are still effective against these organisms. The presence of a carbapenemase in this species cannot be ignored as mutations may occur, leading to a higher level of expression of these enzymes, resulting in treatment failure.

1.9.6.3. *Bacillus* spp.

Bacillus cereus is often regarded as a contaminant or as a non-pathogenic organism when isolated from clinical infections. There are thus relatively little data on its pathogenic role or frequency in clinical infections. The *B. cereus* II metallo- β -lactamase was the first metallo-enzyme reported [164] and the only one for which a crystal structure has been determined [117]. This enzyme has become the 'model' metallo- β -lactamase, although this may not be entirely justified, as this metallo- β -lactamase has certain biochemical properties which differ from most other metallo-enzymes studied to date [103] (this thesis).

1.9.6.4. *Bacteroides* spp.

Most anaerobic infections are polymicrobial and thus require treatment with agents active against an array of aerobic and anaerobic bacteria. Organisms from the *Bacteroides fragilis* group are the most frequently isolated gram negative anaerobic pathogens from clinical infections.

There have been several reports in the last six years in which alterations in the classification of *Bacteroides* have been described. Shah and Gharbia have reported these changes, and related these species to specific sites of colonisation [165].

The frequency of β -lactamase production by various *Bacteroides* species is high. It has been reported that between 75% - 100% of strains from the *Bacteroides fragilis* group are β -lactamase producers [166, 167]. These enzymes are usually serine active chromosomal cephalosporinases, and the species specific cephalosporinase from *Bacteroides uniformis* has been sequenced [168].

There have been many surveys studying the antimicrobial susceptibilities of *Bacteroides* isolates around the world, although there have been few conducted in Great Britain [169-171]. Resistance to carbapenems has been found to vary from 0.1% to 4% [157, 172] even between surveys in the same country, for example France [172, 173] and Japan [174, 175]. This may well be caused by the use of different media and different breakpoints in these surveys, with relatively few studies adhering to the NCCLS guidelines on susceptibility testing of these organisms [176]. This means that many of these surveys cannot be compared directly. In an attempt to circumvent this, several groups have published ongoing susceptibility surveys which, whilst not necessarily conforming to NCCLS guidelines, do at least employ a consistent methodology from year to year [172, 174, 175, 177].

There have been several reports of carbapenemases from isolates of the genus *Bacteroides*, which include metallo- β -lactamases [see Table 1.4] and a carbapenemase whose mechanism of action has not been determined, but is likely to be a serine active site enzyme [157] [see Table 1.5]. One of the metallo- β -lactamases was found to be plasmid mediated and could be transferred to other *Bacteroides* isolates but not to *Escherichia coli* [105] leading to the possible dissemination of this gene into other strains. A report has also been made indicating that many β -lactam sensitive organisms of the *Bacteroides fragilis* group may harbour silent carbapenemase genes which can be activated after exposure to carbapenems [178] or introduction of an insertion sequence [179] resulting in a 100-fold increase in MIC to these compounds. These observations may indicate a potential threat to the use of carbapenems for anaerobic infections. It can be concluded from these reports that resistance to carbapenems may be higher than actually found in many surveys.

1.9.6.4.1. Other Antimicrobials Active Against *Bacteroides* spp.

Tetracyclines were once the drug of choice for *Bact. fragilis* but up to 50% of strains are now resistant, possibly due in part to the presence of an active class F efflux mechanism determinant [180]. Fluoroquinolones show very limited activity against *Bacteroides* and other anaerobes which are regarded as resistant to all but the newest fluoroquinolones [181]. Only *Clostridium perfringens* and some eubacteria show some sensitivity to these compounds. Typical MIC values for *Bact. fragilis* and other members of the *Bact. fragilis* group range from 4-32mg/l [182, 183].

Metronidazole and its hydroxy metabolite [184] are effective against virtually all anaerobes including some anaerobic cocci. However, it has virtually no effect on aerobic bacteria although some bactericidal activity on dormant cells of *Mycobacterium tuberculosis* has been demonstrated recently [185].

The exact mechanism of action of metronidazole *in vivo* is not known, but it has been proposed that this compound and its hydroxy metabolite interact with DNA, preferentially with adenine or thymidine [186], and cause oxidative damage to pyrimidines of metronidazole complexes based on the induction of exonucleases [187]. *In vitro* work involving metronidazole has shown that it interacts with DNA causing single and double strand breaks [188].

It was originally postulated that resistance to 5-nitromidazole antibiotics (such as metronidazole, ornidazole and tinidazole) was not possible, but several *Bact. fragilis* isolates have been described which are resistant to metronidazole [188-190]. 5-nitromidazole resistance has been found to be both plasmid mediated and chromosomally encoded, but was found to be transferable in all isolates tested. Transferability was not tested in one of these strains [189]. One metronidazole resistant *Bact. fragilis* isolate has been found to produce MetA, an extra cellular protein which is able to protect cells against the action of metronidazole by reducing the degree of DNA damage, but has been shown not to inactivate the compound itself [188]. It is unlikely that MetA interacts with DNA since it contains no DNA binding motif, and its mechanism of protection is unknown.

1.9.6.5. *Enterobacter cloacae*.

To date, two similar serine active site (Ambler class A) carbapenemases have been reported [101, 160], but no transfer of resistance was observed. Whilst these enzymes are capable of hydrolysing carbapenem antibiotics, β -lactamase inhibitors such as clavulanic acid still retained some inhibitory activity against these enzymes. This allowed β -lactam / β -lactamase inhibitor combinations to still show some antibacterial activity against organisms producing these β -lactamases.

1.9.6.6. *Flavobacterium* spp.

Bacteria from the genus *Flavobacterium* are widely distributed in natural ecosystems as well as clinical specimens, most notably in neonatal meningitis, and are also recognised as nosocomial pathogens. These organisms show optimal growth at 30°C, but several clinical isolates have been shown to be able to grow at 37°C. As a result of this, their clinical relevance as pathogens is often underestimated.

The first metallo- β -lactamase from this species was identified in 1985 [144]. Recently, two successive *Flavobacterium meningosepticum* isolates from the same patient have been shown to produce an enzyme with carbapenem hydrolysing activity [191], although it is possible that both isolates are in fact the same strain since these strains had identical properties and antibiograms. This report proposed that these enzymes were metallo- β -lactamases solely on the basis of partial inhibition in the presence of EDTA, but this evidence is not conclusive as serine active carbapenemases have been described which are sensitive to EDTA [102, 156]. No other biochemical data on the carbapenemases were given. It is, for these reasons, that this report is not included in Table 1.4. The only other report of a β -lactamase from this species described an extended spectrum β -lactamase from an isolate of *F. meningosepticum* which was unable to hydrolyse imipenem [192].

1.9.6.7. *Pseudomonas* spp.

Pseudomonas aeruginosa has become an important human pathogen because of its innate resistance to many antibiotics. It has been implicated in many nosocomial infections including burns and septicaemia, and is frequently isolated from neutropenic patients.

In a recent survey, the antimicrobial susceptibility of 1991 *P. aeruginosa* isolates were examined. 1.2% of these organisms were found to be resistant to imipenem by NCCLS recommended breakpoint, as opposed to the low breakpoint adopted by that study [193]. A similar study was conducted in 1982, but direct comparison of these is not possible since neither imipenem or meropenem were used in the earlier study [194], and so it has not been possible to determine any trend in the resistance of these organisms to carbapenem antibiotics. One *P. aeruginosa* clinical isolate which produced a plasmid mediated metallo- β -lactamase has been reported [104] but this plasmid was not transferable to *E. coli*.

Burkholderia cepacia, and to a lesser extent *P. aeruginosa* are important pathogens in cystic fibrosis patients. It has been suggested that all *Burk. cepacia* organisms may have the ability to produce carbapenemases [195], and if this holds true, has serious implications for patients with this condition. The metallo- β -lactamase of one such strain has already been characterised [146].

1.9.6.8. *Serratia marcescens*.

Serratia marcescens is becoming more frequently isolated from immunocompromised or Intensive Therapy Unit (ITU) patients. *Serratia marcescens* is one of the few species from which both serine active site carbapenemases [102, 156] and metallo- β -lactamases [106, 147] have been reported. This has serious ramifications for the treatment of this species with carbapenems, as plasmid mediated metallo- β -lactamases have been successfully transferred from this species [106].

1.9.6.9. *Stenotrophomonas maltophilia*.

Recently, this organism has undergone several taxonomic changes, from *Pseudomonas maltophilia* to *Xanthomonas maltophilia*, and most recently to *Stenotrophomonas maltophilia* [196]. The name *Stenotrophomonas* will be used throughout this thesis.

This organism is responsible for septicaemia, meningitis, peritonitis and endocarditis, especially in immuno-compromised patients.

The L1 type metallo- β -lactamase from *Sten. maltophilia* has been shown to be able to hydrolyse imipenem and several penicillins [151]. In addition to L1, *Sten. maltophilia* isolates also produce a cephalosporinase, designated L2 [197]. Both of these enzymes are chromosomally encoded and inducible [151, 197] although the level of induction varies considerably between isolates. The metallo- β -lactamase (which preferentially hydrolyses penicillins and carbapenems) and the cephalosporinase (which hydrolyses cephalosporins) exhibit complementary spectra of activity which together are able to confer resistance to virtually all β -lactam antibiotics. It has been suggested that there is some heterogeneity between the β -lactamases produced by this species [154, 155]. This was supported by the surprising observation that 1mM EDTA did not affect carbapenem hydrolysis in certain *Sten. maltophilia* clinical isolates [154], and more work would be necessary to determine the type of enzyme responsible for the carbapenem hydrolysis in that study.

The observation that many *Sten. maltophilia* isolates show poor membrane permeability to antimicrobials [198], coupled with the production of β -lactamases capable of hydrolysing every class of β -lactam, only reinforces this organism's resistance profile. One study found that *Sten. maltophilia* represented approximately 2% of all nosocomial respiratory tract infections in the laboratory [198], confirming that this organism is emerging as an important nosocomial pathogen.

1.10. Concluding Remarks.

It must be noted that any infection may be polymicrobial and β -lactam treatment failure may be caused by the presence of a metallo- β -lactamase producing organism as part of the mixed clinical infection conferring protection to a more pathogenic organism by hydrolysing the β -lactam agent administered for its treatment.

Metallo- β -lactamases represent a significant threat to the use of β -lactam antibiotics as they have a very broad spectrum of activity, hydrolysing penicillins and cephalosporins as well as carbapenems, and are resistant to classic β -lactamase inhibitors. Their role in the clinical environment is unclear as the vast majority of metallo- β -lactamases studied to date are chromosomally encoded, and not transferable. It is interesting to note that metallo- β -lactamase production has been found predominantly in organisms which until recently have not been considered serious pathogens, for example *Acinetobacter*, *Bacillus cereus*, *Flavobacterium*, *Legionella*, *Serratia* and *Stenotrophomonas*. It is possible that the use of newer and more powerful antimicrobials like the carbapenems have selected these organisms as pathogens as they are able to colonise sites which have been de-nuded of other micro-organisms by the intense antimicrobial activity of these compounds. Many of these patients in ITUs are immuno-compromised, and hence less able to fight these infections themselves.

The rate of dissemination of these carbapenemases into the bacterial population currently appears to be slow, and this is because all but four of these enzymes examined to date have been chromosomally encoded and non-transferable. Carbapenem therapy in Britain is currently restricted to ITU patients, although their use in Europe and South America is less restricted.

The controlled use of carbapenems as 'last line' treatments will slow the spread of these resistance determinants, although such spread is likely to occur in the future as the use of these compounds becomes more widespread.

Aims of This Thesis.

1. To determine the efficacy of the carbapenems and fourth generation cephalosporins amongst random clinical isolates of both aerobic and anaerobic bacteria from clinically important species, and conduct the *in vitro* susceptibilities of these isolates on NCCLS [176] and BSAC [199] recommended agars.
2. To examine the mechanism of resistance in any imipenem resistant strains encountered, with special emphasis on those isolates in which the resistance was mediated by the production of a carbapenem hydrolysing β -lactamase.
3. To examine the assay conditions employed to study metallo- β -lactamases.
4. To determine the incidence of carbapenemase production amongst isolates from the genus *Flavobacterium*, from which only a single metallo- β -lactamase has been previously reported.

CHAPTER TWO

Materials & Methods.

2.1. Chemical Reagents.

All chemical reagents were obtained from Sigma Chemical Co. (Poole, Dorset, UK) unless otherwise stated.

Antibiotics were obtained from the companies listed in Table 2.1 below, and were stored as sterile powders in the absence of light at 4°C until required.

Table 2.1: Source Companies for Antibacterial Compounds.

Compound	Source
Ampicillin	Sigma Chemical Co.
Aztreonam	E.R. Squibb & Sons
Benzylpenicillin	Sigma Chemical Co.
BRL 42715	SmithKline Beecham
Carbenicillin	SmithKline Beecham
Cefotaxime	Roussell Lab. Ltd
Cefoxitin	Merck Sharp and Dohme
Ceftazidime	Glaxo Group Research
Cefuroxime	Sigma Chemical Co.
Cephaloridine	Glaxo Group Research
Ciprofloxacin	Bayer (UK) Ltd
Clavulanic Acid	SmithKline Beecham
Gentamicin	Nicholas Laboratories
Imipenem	Merck Sharp and Dohme
Meropenem	Zeneca Pharmaceuticals
Nitrocephin	SmithKline Beecham
Rifampicin	Ciba Laboratories
Sulbactam	Pfizer pharmaceuticals
Tazobactam	Lederle Laboratories

All antibiotic solutions were prepared immediately prior to use, and were prepared in sterile distilled water for MIC determinations, or prepared in the appropriate assay buffer and maintained at 0°C on ice for spectrophotometric assays.

2.2. Bacterial Strains.

Table 2.2 below describes the source of standard β -lactamases and standard strains which feature in this thesis.

Table 2.2: Bacterial Strains Producing Known β -lactamases.

Species	β -lactamase (pI)	Plasmid	Reference
<i>Bact. fragilis</i> ED-262	Cfi-A-type	Chrom	D.J. Payne Pers. Comm
<i>Bact. fragilis</i> TAL-2480	CfiA (4.6-5.0)	Chrom	[142]
<i>Ent. cloacae</i> NOR-1	Nmc-A (6.9) + AmpC (~9.0)	Chrom	[101]
<i>E. coli</i> K12 J62-2	TEM-1 (5.4)	R1	S.G.B. Amyes
<i>E. coli</i> J53-2	SHV-1 (7.6)	R1010-6	S.G.B. Amyes
<i>Sten. maltophilia</i> ULA-511	L1 (6.9) + L2 (8.4)	Chrom	D.J. Payne

Chrom - Chromosomal β -lactamase.

Pure preparations of the *B. cereus* II and *Sten. maltophilia* L1 metallo- β -lactamases featured in Chapter 7 were received from Dr. D. J. Payne, and had been purified previously.

In addition to the strains in Table 2.2 which produce known β -lactamases, the standard strains listed in Table 2.3 overleaf were also used for comparative and control purposes.

Table 2.3: Control Organisms.

Species	Reference	β -lactamase	Source
<i>Bact. fragilis</i>	NCTC 9343	<i>bla</i> ⁻	NCTC, London
<i>P. aeruginosa</i>	NCTC 10662	<i>bla</i> ⁻	NCTC, London

The clinical isolates examined in this thesis were received from a variety of sources. Certain isolates were received because they exhibited reduced sensitivity or resistance to imipenem. Other isolates were random, and no prior data on their sensitivity to imipenem had been determined. Table 2.4 below details these isolates, and their source, together with the reason for isolation.

Table 2.4: Clinical Isolates Examined in This Thesis.

Species	Number of Isolates	Source	Reason for Selection
<i>Acinetobacter</i> spp.	9	CBL, Edinburgh	Random
<i>Bact. fragilis</i>	1 [¶]	SARL, Edinburgh	Imp ^R
	2 [§]	PHLS, Cardiff	Imp ^R
<i>Bacteroides</i> group	166	CBL, Edinburgh	Random
<i>Burk. cepacia</i>	3	WGH, Edinburgh	Imp ^R
<i>Ent. aerogenes</i>	1	GRI	Imp ^R
<i>Ent. cloacae</i>	1	WGH, Edinburgh	Imp ^R
<i>Enterobacter</i> spp.	9	CBL, Edinburgh	Random
<i>Flavobacterium</i> spp.	10	NCTC, London	Unknown ⁵
<i>Klebsiella</i> spp.	11	CBL, Edinburgh	Random
<i>P. aeruginosa</i>	4	GRI	Imp ^R
	11	WGH, Edinburgh	Imp ^R
<i>Sten maltophilia</i>	18	WGH, Edinburgh	Imp ^R

CBL, Edinburgh - Clinical Bacteriology Laboratories, Edinburgh.

GRI - Glasgow Royal Infirmary.

SARL, Edinburgh - Scottish Anaerobe Reference Laboratory, Edinburgh.

WGH, Edinburgh - Western General Hospital, Edinburgh.

Imp^R - Resistant to Imipenem.

Random - Random Isolate.

Unknown - Reason for submission to NCTC unknown. NCTC catalogue showed no data on imipenem sensitivity.

¶ Strain received was designated JMS-121 [see Chapter 6]. The imipenem resistance of this strain had been previously reported [189].

§ Strains received were designated JMS-219 and JMS-221 [see Chapter 6].

⁵ Each of these strains were distinct clinical isolates, although the reason for submission to the NCTC is not known.

All other strains originate in this thesis unless otherwise stated. The identity of the clinical isolates were confirmed with the identification strips detailed in Table 2.5 below irrespective of their source with the exception of the isolates received from the Clinical Bacteriology Laboratories, Edinburgh which were identified by the staff at the Clinical Bacteriology Laboratories.

Table 2.5: Bacterial Identification Strips.

Suspected Species	Strip type	Manufacturer
<i>Bacteroides fragilis</i>	API32A	Bio Merieux, France
<i>Burkholderia</i> <i>Pseudomonas</i> <i>Stenotrophomonas</i>	API20NE	Bio Merieux, France
<i>Enterobacter</i>	API20E	Bio Merieux, France
<i>Flavobacterium</i>	ATB32GN	Bio Merieux, France

All identification strips were used in accordance with the manufacturers instructions with the appropriate reagents supplied.

2.3. Growth and Storage Media.

All growth media were sterilised by autoclaving at 121°C for 15 minutes at 15 pounds per square inch. Stock solutions of 10mls 50% v/v glycerol were autoclaved twice and stored at room temperature until required. All anaerobic liquid media were pre-reduced overnight in an anaerobic cabinet or anaerobic jar after autoclaving.

All strains were stored in duplicate 1.6ml aliquots at -70°C in equal volumes of growth broth and sterile 50% glycerol.

All media were obtained from Oxoid (Basingstoke, Hants) as dry powders, and prepared as single strength solutions as recommended by the manufacturer unless otherwise stated.



2.3.1. Preparation of Gentamicin Blood Agar (GBA) Plates.

GBA plates consisted of single strength sterile Columbia agar to which was added defibrinated horse blood together with gentamicin to give final concentrations of 5% and 10mg/l respectively, once the molten agar had cooled to below 55°C.

2.3.2. Other Complex Media.

In addition to gentamicin blood agar plates, Isosensitest (IST), MacConkey agars were also used, and prepared according to the manufacturers instructions.

Brain Heart Infusion (BHI) broth, Nutrient broth No.2, thioglycollate broth and Wilkins-Chalgren broth were used, and were obtained as dry powders from Oxoid (Basingstoke, Hants) and prepared as single strength broths. Robertson's cooked meat medium (RCM) was prepared as BHI broth with the addition of 5% v/v dried cooked meat [200].

2.4. In vitro Sensitivity Testing (MIC Determinations).

In each case, a small scale overnight culture [see Section 2.5] of the strains to be tested were diluted in single strength Davis and Mingioli salts (DM) [201]. Single strength DM was prepared as a double strength solution and diluted with an equal volume of sterile distilled water.

Table 2.6: Davis and Mingioli Salts Solution.

Compound	Double Strength (g/l)	Single Strength (g/l)
di Potassium hydrogen phosphate	14	7
Potassium hydrogen phosphate	6	3
tri-Sodium citrate	0.94	0.47
Magnesium sulphate	0.2	0.1
Ammonium sulphate	2	1

2.4.1. Aerobes.

Sensitivity testing of all aerobic isolates was performed according to the NCCLS guidelines [176] where applicable on Oxoid Isosensitest (IST) agar (Oxoid, Basingstoke, Hants.) containing doubling dilutions of the antimicrobial agent. A 2µl inoculation was achieved with a Denley multipoint inoculator (Denley, Sussex) at 10^4 cfu per spot, except *Flavobacterium* strains which were inoculated at 10^6 cfu per spot unless otherwise stated. Plates were incubated for 16 hours at 37°C for all isolates except those belonging to the genus *Flavobacterium* which were incubated for 16 hours at 30°C.

2.4.2. Anaerobes.

Sensitivity testing of *Bacteroides* isolates was performed according to the NCCLS guidelines [176] on Wilkins-Chalgren agar (Oxoid) supplemented with haemin [202] and 5% defibrinated horse blood [203] containing doubling dilutions of the antimicrobial agent. Inoculation was achieved with a Denley multipoint inoculator at 10^4 cfu per spot. Plates were incubated for 48 hours at 37°C in either an anaerobic cabinet or an anaerobic jar with an Oxoid BR38 Gas Generating Kit. A single plate inoculated as above but without antibiotic was also incubated aerobically at 37°C for 16 hours to ensure that no aerobic contaminants were present.

In all cases, the MIC was taken as the lowest concentration of the selected antimicrobial which effected significant inhibition of growth. Where the MICs of β -lactam / β -lactamase inhibitors were determined, for example amoxycillin and clavulanic acid, the concentration stated is that of the β -lactam, and all combinations were tested in a 2:1 ratio unless otherwise stated.

Antibiotic sensitivity discs were supplied by Mast Laboratories Ltd (Merseyside).

2.5. Culture of Clinical Isolates.

2.5.1. Aerobes.

Small scale cultures were grown in 10mls Oxoid Nutrient Broth No. 2 in Universal bottles and incubated for 16 hours at 37°C with agitation at 200 rpm. *Flavobacterium* isolates were cultured in the same broth, but incubated at 28-30°C with agitation wherever possible. Strains were tested for β -lactamase induction by the addition of imipenem after 2 hours growth to a final concentration of $\frac{1}{4}$ the MIC of the cultured strain [204].

Large scale cultures of aerobic bacteria were initially grown as small scale cultures, and a 1ml inoculum was added to 1 litre of pre-warmed Nutrient Broth No. 2 in 2 litre conical flasks, or multiples thereof. Imipenem was always added to a final concentration of $\frac{1}{4}$ the MIC of the cultured strain, whether inducible or not, to ensure β -lactamase production.

2.5.2. Anaerobes.

Small scale cultures were set up by taking single colonies from a GBA plate and inoculating 20mls Robertson's Cooked Meat medium in McCartney bottles.

Large scale cultures were initially set up as small scale cultures as described above. 10mls of this small scale culture was added to 1 litre of Robertson's Cooked Meat Broth in a 1 litre Duran bottle which had been pre-reduced and pre-warmed to 37°C. Imipenem was added to a final concentration of $\frac{1}{4}$ the MIC of the strain on Wilkins Chalgren agar or 16mg/l, whichever was lower. The culture was allowed to grow overnight at 37°C.

2.6. β -lactamase Preparation.

The stationary phase culture was harvested at 6000 x *g* at 4°C for 20 minutes in a Sorvall RC-5B refrigerated centrifuge or a Beckman J2-21 Refrigerated Superspeed centrifuge. This force was increased to 7000 x *g* for *Flavobacterium* isolates. The pellets were resuspended in 25mM PIPES buffer pH 7.0 supplemented with 1mM ZnSO₄ (unless otherwise stated) as a washing step, and re-spun.

Pellets were resuspended in the minimum volume of 25mM PIPES pH 7.0 supplemented with 1mM ZnSO₄ (unless otherwise stated) and sonicated (MSE SoniPrep 150) on ice at an amplitude of 8 μ M for 3 x 30 seconds with 30 second intervals for aerobes and 5 x 30 seconds with 30 second intervals for anaerobes.

The resulting lysate was centrifuged at 40,000 x *g* for 60 minutes for all strains with the exception of *Flavobacterium* isolates which were centrifuged at 60,000 x *g* in an MSE Europa or a Beckman refrigerated ultracentrifuge.

The increased *g* force for all *Flavobacterium* centrifugation was required to obtain satisfactory separation of the pellet and supernatant.

2.7. Analytical Iso-electric Focusing.

β -lactamases were separated and identified by analytical iso-electric focusing (IEF) on a thin layer polyacrylamide gel film as described by Matthew [205] containing broad range carrier ampholytes.

Two methodologies were employed for this procedure; one involving the making of the gel pH 3.5-10 followed by polymerisation, and the other involving pre-cast Pharmacia Ampholine PAGplate gels pH 3.5 to 9.5. These methods are described separately below:

2.7.1. Preparation of IEF gel pH 3.5-10.

The poly-acrylamide gel was made as described in Table 2.7 below.

Table 2.7: Composition of Analytical IEF Gels.

Solution	Volume in gel (ml)	Final concentration
Sterile distilled water	25	-
5% v/v N,N,N',N'-tetramethyl-ethylenediamine (TEMED)	0.2	0.05% v/v
40% w/v carrier ampholytes: pH3.5-10	2.0	2% w/v
33.9%T, 2.7%C acrylamide/ N,N'methylene bis-acrylamide	9.0	7.6%T 2.7%C
0.002% w/v Riboflavin	4.0	0.0002% w/v

The gel solution was poured between two glass plates of differing sizes, the smaller of which was coated to promote the adhesion of the gel. The plate coating solution consisted of 0.5% w/v gelatin and 0.5% w/v chromium potassium sulphate in sterile distilled water, and was allowed to dry on the plate. When poured, the acrylamide was polymerised by the riboflavin in the presence of ultraviolet light for 6 hours, forming a gel measuring 200mm x 150mm x 1mm.

When the glass plates were prised apart, the gel (which had adhered to the smaller plate) was allowed to dry in air for up to 60 minutes before loading with samples of β -lactamase preparations on to the gel surface close to the anode. The nitrocephin spot test time determined the volume of the β -lactamase preparation loaded onto the gel, the volume being calculated as the nitrocephin spot test time (in seconds) [Section 2.10.1] up to a maximum of 30 μ l for crude cell extracts and 100 μ l for partially purified β -lactamases.

The IEF gels were focused overnight at 4°C with the following limiting parameters; voltage = 500V, current = 20mA, power = 1.0W. The gels were calibrated by focusing β -lactamases of known pI.

2.7.2. Pre-cast IEF Gels.

Pre-cast gels had a range of pH 3.5 to 9.5 (T=5%, C=3%) (PAGplate, Pharmacia, Sweden) and up to 70 μ l of the sample to be focused was loaded onto applicator strips placed near the anode, the exact volume being determined by the nitrocephin spot test time as described in Section 2.10.1. The only modification to the manufacturers instructions was that the filter paper electrode strips between the electrodes and the gel were soaked in distilled water as opposed to the H_3PO_4 and NaOH recommended. The gel was laid on a ceramic plate which was water cooled and maintained at 9°C. The gel was focused for 3 hours with the following limiting parameters; voltage = 1500V, current 50mA, power = 30W. The gels were calibrated by loading 5 μ l IEF markers pI 4.7-10.6 (BDH, Poole, Dorset).

The focused β -lactamases on both gel types were visualised firstly by overlaying the gel with a sheet of Whatman No. 1 filter paper soaked in 1mM $ZnSO_4$ for five minutes, followed by overlaying the gel with filter paper soaked in 500mg/l nitrocephin. The iso-electric points of unknown or novel enzymes were estimated from the focused bands of β -lactamases of known pI or iso-electric focusing makers.

2.8. Partial Purification of β -lactamases.

2.8.1. Gel filtration.

Sephadex (Pharmacia, Sweden) was used for molecular mass estimations and partial β -lactamase purification as described by Andrews [206]. Sephadex consists of beads of controlled diameter, the bead size determining the range of molecular masses of peptides and globular proteins which can be fractionated by the column. Both G75 (useful range 10-50kDa) and G150 (useful range 20-150kDa) were used.

2.8.1.1. Column Preparation.

Dry Sephadex, either G75 or G150 was taken and swollen in 25mM PIPES buffer pH 7.0 supplemented with 1mM ZnSO_4 , a volume 15 times that of the dry mass of the Sephadex, and steamed for 3 hours. Once steamed, the slurry was cooled to 4°C and slowly poured into a vertical glass column 2cm² x 90cm (Amicon Ltd, Glos, UK.), allowing the slurry to pack together. The packed column was washed for 48 hours with the same buffer at a flow rate of 12mls/hour. The buffer was pumped up the column against gravity by a P-1 peristaltic pump (Pharmacia). The column was maintained at 4°C with continuous recycling of the column buffer in a reservoir containing at least 500mls of the same buffer.

2.8.1.2. Calibration.

The column was calibrated by the addition of a 1ml solution of column buffer containing 10mg of each of three proteins of known molecular mass which, in the case of a G75 column, were ovalbumin (47kDa), chymotrypsinogen A (25.5kDa) and cytochrome C (12384Da). These proteins were separated as they passed through the column, and were collected by an LKB-BROMMA 2070 fraction collector (Pharmacia, Sweden) in 2ml aliquots. The relative elution volumes of these proteins were determined by measuring the A_{280} of the fractions on a Perkin Elmer Lambda 2 UV/Vis Spectrophotometer

(Bucks, UK). The void volume of the column was determined by the addition of 1ml of 0.1% w/v Blue Dextran ($M_r \sim 2\text{MDa}$) as described above for calibration. A plot of molecular mass against elution volume could be generated, forming a standard graph from which the molecular mass of a β -lactamase could be determined as outlined in Figure 2.1 below and Figure 2.2 overleaf.

Figure 2.1: Typical Elution Profile of Known Proteins from a Sephadex G75 Column.

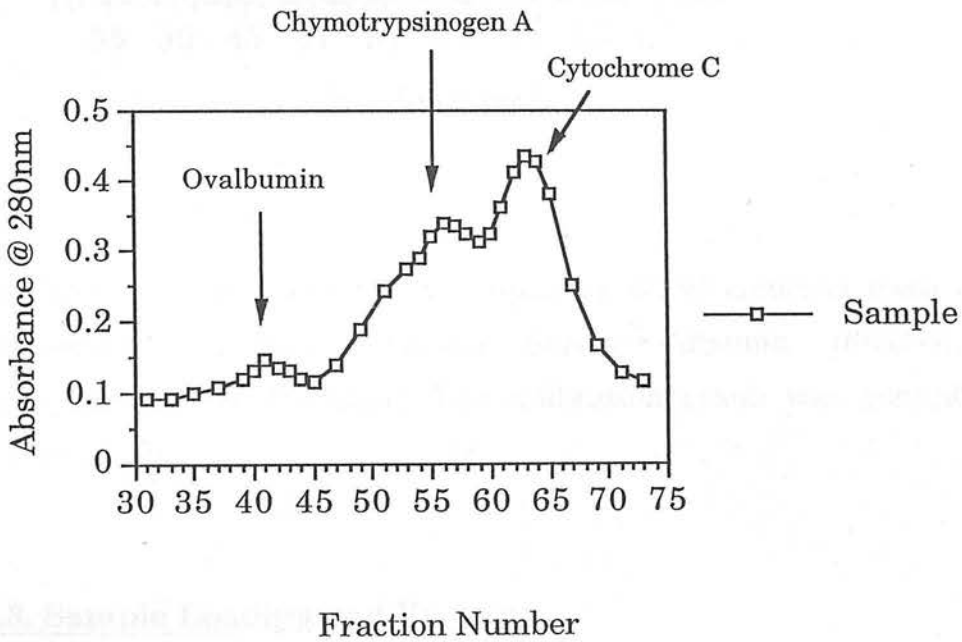
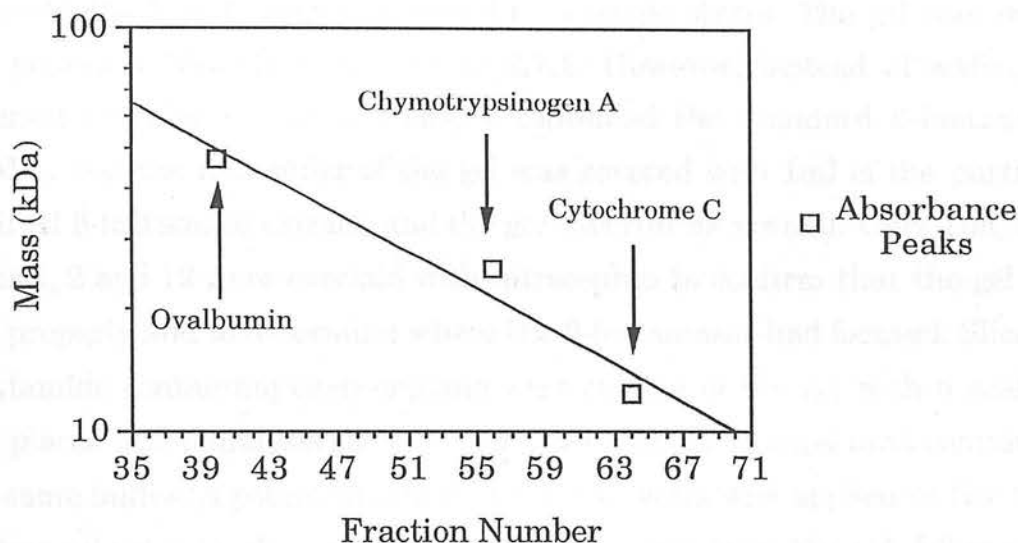


Figure 2.2: Typical Semi-Log Calibration Plot of Known Proteins from a Sephadex G75 Column.



The molecular mass standards for Sephadex G150 columns were alcohol dehydrogenase, (150kDa), Bovine Serum Albumin (66kDa), and chymotrypsinogen A (25.5kDa). The calibration graph was generated as described above.

2.8.1.3. Sample Loading and Running.

Crude cell free lysates were added to the column at a flow rate of 12mls/hour in a volume no greater than 2mls, and eluted with the column buffer, ensuring that 2ml fractions from least one column volume were collected. Fractions containing β -lactamase activity were initially determined by nitrocephin spot test, and then assayed on a Perkin Elmer Lambda 2 spectrophotometer for both nitrocephin hydrolysing activity (ΔAbs @ 384nm) and imipenemase activity (ΔAbs @ 299nm).

2.8.2. Preparative Iso-electric Focusing of β -lactamases.

Preparative IEF of partially purified β -lactamases was employed to separate the β -lactamases produced by a single strain. The gel was made and poured as described in Section 2.7.1. However, instead of adding 12 different samples to the gel, lane 1 contained the standard β -lactamase TEM-1, and the remainder of the gel was covered with 1ml of the partially purified β -lactamase extract, and the gel was run as normal. Once run, only lanes 1, 2 and 12 were overlaid with nitrocephin to confirm that the gel had run properly and to determine where the β -lactamases had focused. Slices of acrylamide containing each enzyme were cut out of the gel with a scalpel, and placed into a dialysis sac with 5ml PIPES buffer in a gel tank containing the same buffer. A potential difference of 150 volts was applied to the tank for two minutes to allow the β -lactamase to elute from the gel, followed by 20 seconds with the polarity reversed to ensure that the enzyme did not remain bound to the side of the sac. The buffer within the sac was collected and assayed for β -lactamase activity.

2.8.3. β -lactamase Purification by Fast Protein Liquid Chromatography (FPLC).

2.8.3.1. Optimum Matrix determination.

A screening system involving 46 different ion exchange matrices from all major matrix groups was used to determine the most effective matrix for β -lactamase purification. Table 2.8 overleaf lists the matrices tested.

Table 2.8: Matrices Tested for Optimum β -lactamase Binding.

Number	Matrix	Number	Matrix
1	Sepharose CL4B (control)	24	Green 1 Agarose
2	Gelatin Sepharose	25	Blue 1 Agarose
3	Heparin Sepharose	26	Blue 2 Agarose
4	Fibronectin Sepharose	27	DEAE Sepharose
5	Bacitracin Sepharose	28	CM Sepharose
6	ω -Amino Hexyl Sepharose	29	Q-Sepharose FF
7	<i>p</i> -ABA Sepharose	30	MacroPrep High Q
8	Lysine Sepharose	31	S-Sepharose
9	Benzamidine Sepharose	32	MacroPrep High S
10	Protein A Sepharose	33	HW 650S (control)
11	Aprotonin Agarose	34	Toyopearl Butyl 650S
12	Zinc Chelate	35	Toyopearl Butyl 650M
13	Prosep A (high capacity)	36	MacroPrep Butyl
14	D-Alanine Agarose	37	Butyl Sepharose FF
15	Blue Sepharose	38	MacroPrep Methyl
16	Green Agarose	39	Phenyl Sepharose (HP)
17	Red 1 Agarose	40	Phenyl Sepharose 6FF (LS)
18	Red 2 Agarose	41	Phenyl Sepharose 6FF (HS)
19	Orange 1 Agarose	42	Octyl Sepharose 4FF
20	Orange 2 Agarose	43	Fractogel EMD SO_3^-
21	Orange 3 Agarose	44	Fractogel EMD TMEA 650S
22	Yellow 1 Agarose	45	Fractogel TSK DEAE 650S
23	Yellow 2 Agarose	46	Hydroxyapatite

HP - High Performance.

LS - Low sub.

HS - High sub.

All matrices were stored in de-ionised water / 0.02% Kathon CG.

100 μ l of each matrix suspension was added into a separate well of a V-bottomed microtitre plate. 50 μ l of the crude cell lysate was added to each well and mixed with the suspension, and then left for 30 minutes at room temperature to facilitate protein binding to the matrix. After this time, the plate was centrifuged at 1000 rpm for 1 minute to pellet the matrix together with bound proteins. 50 μ l of the supernatant from each well was removed to a clean flat-bottomed microtitre plate, and 100 μ moles nitrocephin was added to the supernatant aliquots. The supernatants of matrices which bound the β -lactamase would remain yellow, whilst those which did not bind the β -lactamase turned red as the β -lactamase still present in the

supernatant hydrolysed the nitrocephin. 'Hits' were defined at those matrices whose supernatants remained yellow after the addition of nitrocephin.

Where this matrix screen did not yield any 'hits', small scale columns (bed volume ~20mls) were set up and equilibrated with an appropriate buffer at 2ml/minute. A sample of the crude cell-free β -lactamase extract was applied to the column and allowed to adsorb onto the matrix. Once the A_{280} of the eluent had fallen below 0.05, the proteins were eluted in NaCl step gradients of 0.25M, 0.5M, 0.75M, and 1.0M at 2ml/minute. Each step was continued until the A_{280} of the eluent no longer increased. Step eluted fractions were assayed for β -lactamase activity and subjected to IEF to determine if the column had bound the β -lactamase successfully before a large scale column was prepared.

2.8.3.2. Column Preparation and Running.

Once the matrix had been chosen, a column was poured and mounted onto a Pharmacia FPLC system as detailed in Table 6.2. The column was equilibrated with running buffer which had been degassed.

The pH of the sample which was to be loaded onto the column was altered to that of the running buffer by a 1:4 dilution with running buffer followed by pH adjustment with an acid or base appropriate to the running buffer if necessary. The sample was loaded onto the column at a rate of 2ml/minute. Once loaded, the column was flushed with running buffer until the A_{280} of the column eluent fell below 0.05.

Proteins bound to the column were selectively eluted by an increasing concentration of NaCl in the running buffer, with column eluent collected in 5ml fractions. The concentration of NaCl in the running buffer varied from 0 to 1M, although the gradient was not always linear as detailed in Figures 6.4-6.7. However, in all cases, the run started with no NaCl and finished with 1M NaCl in the buffer, and ran for at least 4 column volumes.

Fractions containing β -lactamase activity were detected by spectrophotometric assays with both nitrocephin and imipenem as reporter substrates. Active fractions were pooled, divided into 1ml aliquots and rapidly frozen on solid CO_2 to -20°C and assayed within one week.

2.8.4. Ion Exchange Chromatography.

This section details the protocol for running a Whatman DE52 anion exchange column. This section refers solely to Chapter 3, and was used to separate NmcA (pI 6.9) and AmpC (pI ~9.0) from *Enterobacter cloacae* NOR-1.

150g (dry mass) of Whatman DE52 DEAE (diethylaminoethyl-) cellulose was prepared according to the manufacturers instructions and equilibrated with 10mM Tris HCl pH 8.5⁶ in a Wright jacketed column (4cm² x 60 cm) at 4°C in a cold cabinet.

The crude dialysed β -lactamase preparation was added to the top of the column. Once the sample had adsorbed to the top of the matrix, the column was flushed with running buffer until the A_{280} of the eluent fell below 0.08.

The β -lactamase was eluted by a linear NaCl gradient, increasing from 0M to 1M NaCl in the running buffer. 5ml samples were collected in a LKB BROMMA 2070 fraction Collector (Pharmacia, Sweden).

2.8.5. Ammonium Sulphate Selective Precipitation.

Crude cell-free lysates containing β -lactamase activity were placed in a beaker and cooled to 4°C. Ammonium sulphate was added slowly, over a course of 30 minutes to a final saturation of 30% with gentle stirring, the

⁶ The change in pKa per degree celcius for Tris buffer is very high ($\Delta\text{pKa}/^\circ\text{C} = -0.031$). For this reason, This buffer was made up and pH altered to 8.5 at 4°C. Otherwise a buffer pH 8.5 made at 25°C and chilled to 4°C will drop by 0.65 pH units to 7.85.

exact mass of ammonium sulphate was determined as described by [207]. After each increase in ammonium sulphate saturation, the solution was left for 30 minutes with gentle stirring. The precipitate was harvested by centrifugation at 15,000 $\times g$ in a Sorvall RC-5B refrigerated centrifuge or a Beckman J2-21 Refrigerated Superspeed centrifuge for 10 minutes. The pellet was resuspended in a minimum volume of 25mM PIPES pH 7.0 with 1mM ZnSO_4 and assayed together with the supernatant for β -lactamase activity by nitrocephin spot test to determine whether the β -lactamase had been precipitated. If not, the saturation of the supernatant was increased to 50% as described above and the precipitated protein was re-assayed. This was increased to a final saturation of 80% ammonium sulphate if necessary. The fractions were dialysed against 25mM PIPES pH 7.0 with 1mM ZnSO_4 to remove all ammonium sulphate, and assayed for β -lactamase activity spectrophotometrically.

2.8.6. Free flow IEF.

Free flow IEF is a technique in which proteins can be separated in a similar manner to conventional IEF, but the pH gradient is set up across a moving liquid containing carrier ampholytes. This allows a larger amount of protein to be loaded, and facilitates the collection of focused proteins of narrow pH ranges (approximately 0.5 pH units).

Crude β -lactamase extracts were purified on the MinipHor system (Rainin, California) which was used according to the manufacturers instructions. Additional solutions are listed in Table 2.9 below.

Table 2.9: Component Solutions for Free-Flow-IEF.

Solution	Volume (ml)	Final Concentration
Pharmalyte pH 3.5-10 (40% v/v)	0.8	1% v/v
Glycerol	3.5	10% v/v
Glycine (100% w/v)	0.35	1% w/v

The solution was made up to a final volume of 30ml with deionised water, and loaded onto the MinipHor apparatus and pre-focused for 30 minutes at 4°C.

Once prefocused, a 5ml crude cell-free extract was injected and allowed to focus at 1000V and 40W limiting for 30 minutes, followed by 500V 20W limiting for a further 10 minutes prior to sample collection. A total of 20 samples of approximately 1.25ml were collected, representing the pH spectrum of the pharmalytes. The exact pH of each sample was determined by pH indicator paper strips (BDH Laboratories). Samples were dialysed against a 100 fold excess of 25mM PIPES pH 7.0 in 1mM zinc sulphate to remove the pharmalytes and then assayed against nitrocephin and imipenem for β -lactamase activity.

2.9. Protein Determination.

The protein concentration of a given sample was determined in one of two ways.

2.9.1. Spectrophotometric.

This method was first described by Waddell [208]. A dilution (1 in 1000) of the sample was placed in a quartz cuvette with a path length of 1cm. The protein concentration was calculated according to the equation $A_{215} - A_{225} \times 165$.

2.9.2. Chemical.

This method was first described by Bradford [209]. 10 μ l of the sample was added to 100 μ l BioRad Coomassie brilliant blue G250 reagent in a microtitre plate, and the A_{600} was compared with a standard graph. The standard graphs were prepared with bovine serum albumin by Dr. P. J. E. Rowling, SmithKline Beecham Pharmaceuticals.

2.10. Assays for β -lactamase Activity.

2.10.1. Nitrocephin Spot Test.

30 μ l of the sample was placed in a well of a microtitre plate, and mixed with 100 μ l of 50mg/l nitrocephin. The time taken for the nitrocephin to be hydrolysed, characterised by a colour change from yellow to red was noted. Where this time was greater than five minutes, the plate was incubated at 37°C.

2.10.2. Kinetic Analysis.

Assays of β -lactamase activity were conducted on a Perkin Elmer Lambda 2 dual beam spectrophotometer or a Beckman DU-7400 diode-array spectrophotometer as described by [210, 211]. Both spectrophotometers had thermostatically controlled cuvettes, and assays were conducted at 37°C in 25mM PIPES pH 7.0 unless otherwise stated. In all cases, the buffer present in the 'blank' cuvette was identical to that present in the test cuvette, including any zinc supplement for the experiments in Chapter 5.

All β -lactamase assays were conducted in the absence of a ZnSO_4 supplement with the exception of those detailed in Chapter 5.

The rates of hydrolysis of nitrocephin were measured at 10^{-4}M . Both carbapenems and cephalosporins were measured at 10^{-3}M , and penicillins were measured at 10^{-2}M . The hydrolysis of β -lactam antibiotics were measured at the wavelengths detailed in Table 2.10 overleaf.

Table 2.10: Wavelengths for Measurement of β -lactam Antibiotics.

Compound	Molecular Mass	λ_{max} (nm)	
		P.E. Lambda 2	Beckman DU7400
Ampicillin	371.4	238	235
Cefuroxime	446.4	260	260
Cefotaxime	477.4	265	260
Ceftazidime	636.6	260	260
Imipenem	321.4	299	299
Meropenem	437.5	300	300
Nitrocephin	516	384	482
BRL 42715	300	-	285
Cefoxitin	449	-	268
Cefpirome	462	260	260
Cephaloridine	415.5	255	264
Ceftriaxone	509.2	-	258
Cefepime	480.6	260	260

P.E. Lambda 2 - Perkin Elmer Lambda 2 spectrophotometer.

The activity of the β -lactamase was determined by the equation:

$$R = \frac{\Delta\text{Abs} \times n \times 1000}{\Delta\text{Abs}_{\text{total}} \times \text{time} \times \text{enz}}$$

Where:

R = μ moles of substrate hydrolysed per minute per ml enzyme.

ΔAbs = 'corrected' change in absorbance measured.

n = μ moles substrate in the cuvette.

$\Delta\text{Abs}_{\text{total}}$ = Total change in absorbance after complete substrate hydrolysis.

time = time in minutes.

enz = volume of enzyme present (μ l).

The corrected change in absorbance describes a subtraction of the change in absorbance in the presence of enzyme and substrate from the change in absorbance in the presence of substrate, but in the absence of enzyme.

Enzyme inhibition experiments were conducted with nitrocephin as reporter substrate unless otherwise stated. ID_{50} s (the concentration of inhibitor required to inhibit hydrolytic activity by 50%) were determined by a five

minute pre-incubation of increasing concentrations of inhibitor (10^{-9}M - 10^{-3}M) with the enzyme at 37°C prior to the addition of substrate unless otherwise stated.

The enzymes were assayed in decreasing substrate concentrations and decreasing rates of hydrolysis plotted on a double reciprocal plot to determine V_{max} and K_{m} as described by Lineweaver and Burk [212].

2.11. Molecular Mass Determinations.

The molecular masses of β -lactamases were estimated in two ways.

2.11.1. Sephadex Gel Filtration.

The eluted fractions of a calibrated sephadex column showing peak β -lactamase activity could be compared to the standard graph as described in Section 2.8.1.2.

2.11.2. SDS PAGE.

2.11.2.1. Sample Preparation.

Samples contained up to $30\mu\text{g}$ protein in up to $30\mu\text{l}$. (which was increased to $40\mu\text{l}$ after the addition of loading reagents). Samples of less than $30\mu\text{l}$ were made up to $30\mu\text{l}$ with sterile distilled water. The reagents described in Table 2.11 were added to the sample before loading.

Table 2.11: Additional Reagents for SDS-PAGE Samples.

Stock solution	Volume (μl)	Final Concentration
10% SDS	4	1% (v/v)
Phenol Red	4	10% (v/v)
β -2 Mercaptoethanol	2	5% (v/v)

This mixture was heated to 70°C for 20 minutes immediately prior to loading onto the gel.

2.11.2.2. Gel Preparation and Running.

Samples were run on precast Novex 10-well polyacrylamide minigels (10-27% acrylamide; 2.6-5% Bis-acrylamide) of the Tris-Glycine type, pH 8.6 which separate proteins between 5-100kDa. Gels were stored at 4°C. The gel and gel wells were rinsed thoroughly in deionised water prior to use to remove any unpolymerised acrylamide and storage solution.

Samples were loaded with a Hamilton Syringe and run in 1x Laemmli buffer at 80mA (constant current) for 80 minutes or until the Phenol Red dye front was just visible at the bottom of the gel.

Once run, the plastic cassette enclosing the gel was opened and the bottom 3mm of the gel and the stacking gel at the top were removed and discarded.

2.11.2.3. Stain / Destain.

The gel was stained in Coomassie Blue R-250 for 20 minutes with gentle shaking, and washed in deionised water.

The gel was destained twice in destain solution (5% methanol, 10% acetic acid, 85% deionised water) for 1 hour with gentle shaking together with foam bungs cut into small pieces to help absorb excess stain.

2.12. Passage Experiments.

2.12.1. Selection of Imipenem Resistant Mutants.

The strains to be passaged were set up in Robertson's Cooked Meat Broth without imipenem.

Broth cultures of the selected strains were set up in 20 ml broth Wilkins Chalgren broth (unless otherwise stated) in McCartney bottles with increasing concentrations of imipenem. A total of six broths were set up for each strain, one bottle a single dilution below the MIC, one bottle equal to the MIC, and three bottles with increasing concentrations of imipenem. One bottle contained no imipenem to act as a control.

The bottles were inoculated with a 1% inoculum of the strain to be passaged unless otherwise stated and incubated for 24 hours at 37°C.

Each day for five days, the bottle with the highest imipenem concentration which still showed significant bacterial growth was used to inoculate 6 fresh bottles with imipenem as before.

2.12.2. Selection of Imipenem Sensitive Mutants.

A culture of the strain was set up in 4.5 ml Nutrient Broth No. 2 with no selective pressure in a universal bottle, and incubated for 24 hours at 37°C. Each day, 50µl was taken and used to inoculate a fresh pre-warmed universal containing 4.5ml broth. This procedure was repeated for 26 days. The antibiograms of the strain and the passaged mutant were compared to determine any change in the level of β -lactam resistance.

2.13. Conjugation Studies.

2.13.1. Formation of Rifampicin Resistant Mutant.

Since no suitable recipient for *Bacteroides* conjugation studies was available, a mutant strain of the β -lactamase negative *Bact. fragilis* NCTC 9343 was selected for the formation of a rifampicin mutant.

This strain was cultured in 100ml Robertson's Cooked Meat medium at 37°C overnight. The culture was decanted into a sterile centrifuge bottle and centrifuged at 6000 x *g* for 20 minutes at 4°C in a Beckman J2-21 Refrigerated Superspeed centrifuge. The pellet was resuspended in 4ml fresh BHI broth, and 1ml was plated onto each of four GBA plates containing 20mg/l rifampicin and incubated in an anaerobic jar for 60 hours. Of the three colonies that grew, all were plated onto fresh GBA plates containing 20mg/l rifampicin for a further 60 hours.

Each of these colonies were identified by API32A, but only was identified as *Bact. fragilis*. This strain was designated JMS-431.

2.13.2. Mating Experiments.

Donor and recipient strains were incubated in 20ml Robertson's Cooked Meat medium in a sealed McCartney bottle overnight at 37°C.

5ml fresh pre-warmed Robertson's Cooked Meat medium was inoculated with 0.1ml donor and 1ml recipient and incubated for 8 hours at 37°C. After this time, the culture was harvested by centrifugation at 6000 x *g* in a Beckman J2-21 Refrigerated Superspeed centrifuge, resuspended in 2ml sterile 0.85% saline and inoculated onto Columbia blood agar plates with 20mg/l rifampicin and 4mg/l imipenem. The plates were incubated anaerobically for 48 hours at 37°C.

CHAPTER THREE

Purification of Known Carbapenemases.

3.1. Prologue.

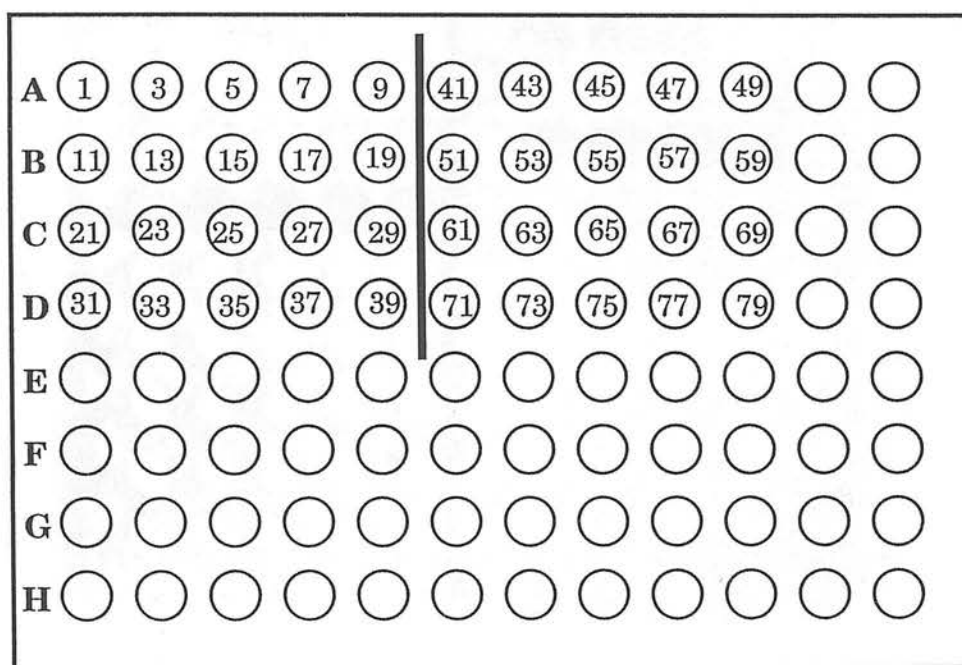
To ensure the accuracy of results obtained in this thesis during experiments on carbapenem hydrolysing enzymes, several previously described carbapenemases were included in these experiments for control and comparative purposes.

Since the experiments involving the effects of divalent cations on carbapenemase activity [see Chapter 5] required a serine active carbapenemase, β -lactamase Nmca was purified from *Enterobacter cloacae* NOR-1.

3.2. Purification of Nmca from *Enterobacter cloacae* NOR-1.

Enterobacter cloacae NOR-1 is known to produce two β -lactamases; a serine active site carbapenemase designated Nmca and an AmpC type chromosomal cephalosporinase. Since these β -lactamases have different iso-electric points, 6.9 and \sim 9.3 respectively, they were separated on a DEAE cellulose column (Whatman DE52) equilibrated in 10mM Tris pH 8.5 as described in Materials and Methods. The AmpC cephalosporinase failed to bind to the column, and was eluted in the void volume. However, Nmca bound to the column matrix, and was eluted by an linear NaCl gradient. A nitrocephin microtitre plate assay of the eluted fractions was conducted to locate those with β -lactamase activity. This plate can be seen in Figure 3.1 overleaf and Figure 3.2.

Figure 3.1: Diagrammatic View of Microtitre Plate Assay To Determine Fractions with β -lactamase Activity After Ion Exchange Chromatography.



The above diagram denotes the loading sequence for the microtitre plate assay. The numbers represent the fraction numbers. The salt gradient was started at fraction 41.

Figure 3.2: Microtitre Plate Assay To Determine Fractions with β -lactamase Activity After Ion Exchange Chromatography.

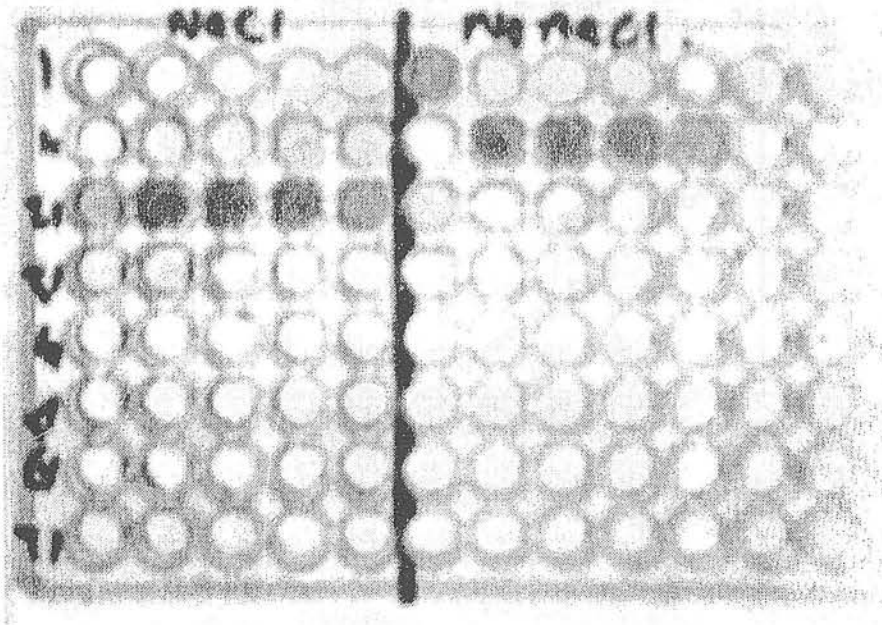


Figure 3.3 overleaf shows the elution profiles of these β -lactamases.

Figure 3.3: Elution Profiles of NOR-1 β -lactamases by Ion Exchange.

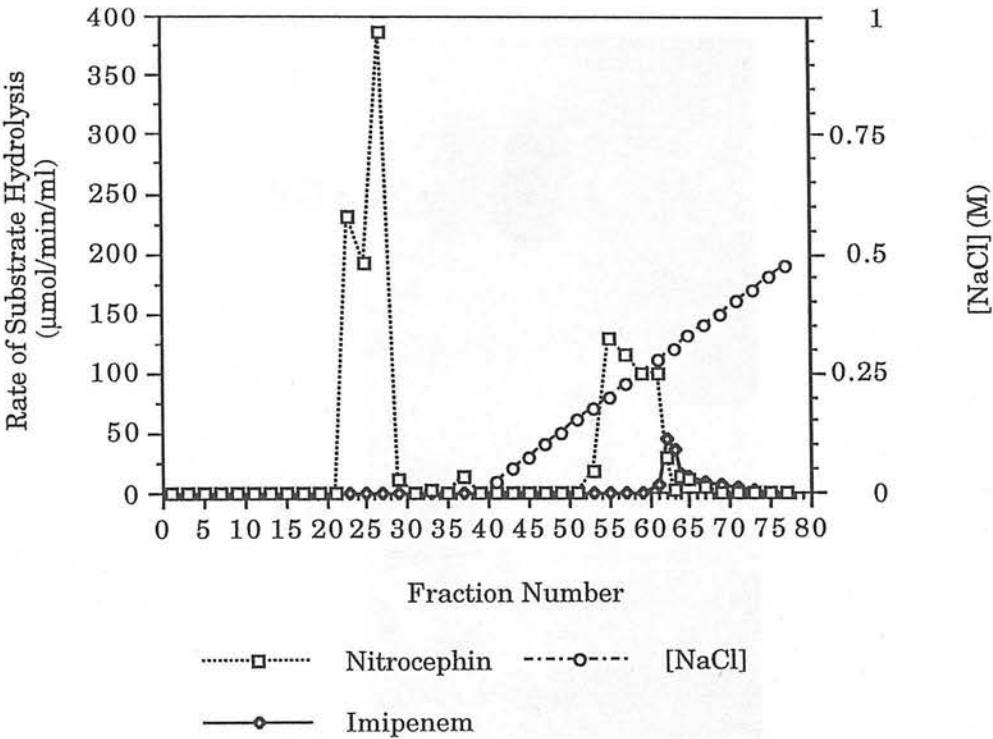
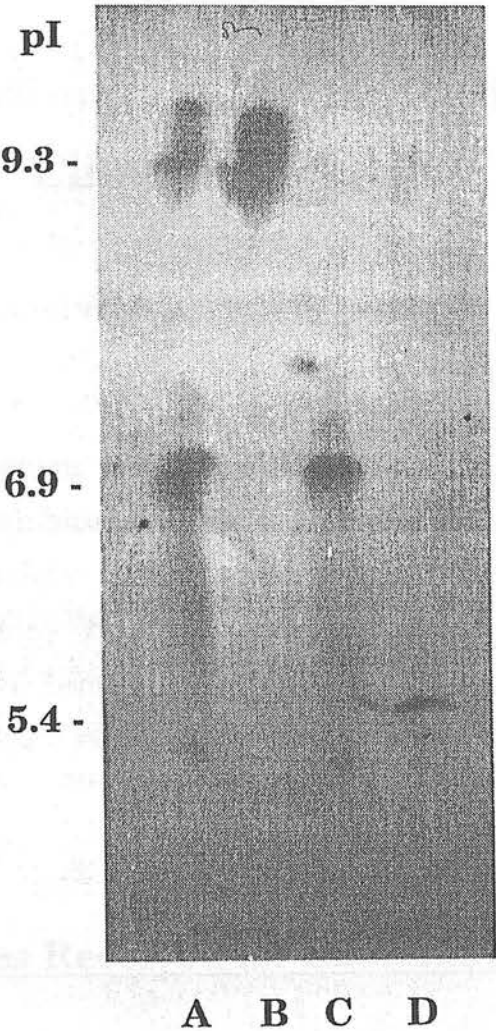


Figure 3.4 overleaf shows an IEF gel with the separated β -lactamases, together with the crude preparation of *Enterobacter cloacae* NOR-1.

Figure 3.4: IEF Showing Separation of *Enterobacter cloacae* NOR-1 β -lactamases by Ion Exchange Chromatography.



Lane A	Crude Extract of <i>Enterobacter cloacae</i> NOR-1.
Lane B	AmpC.
Lane C	Nmc-A.
Lane D	TEM-1.

CHAPTER FOUR

Determination of β -lactam Resistance in Clinical Bacteria.

4.1. Prologue.

Resistance to carbapenem antibiotics is rare, as stated in Chapter 1. Of those strains which exhibit resistance to carbapenems, such resistance can be mediated by a variety of mechanisms, including the production of a β -lactamase which may be capable of hydrolysing these compounds. Carbapenemases have been found which employ a metal ion as part of their active site and more recently, serine active site carbapenemases have also been reported.

4.2. Clinical Isolates Received for Examination.

As part of this thesis, clinical isolates from several clinically relevant species have been collected as described in Section 2.2. This collection has been examined both for carbapenem resistance and for the production of a carbapenem hydrolysing β -lactamase.

These isolates are discussed in the following sections, and classified by source.

4.2.1. Isolates from Glasgow Royal Infirmary.

Five clinical isolates had been identified by Glasgow Royal Infirmary as resistant to imipenem by modified Stokes test. Four of these strains were confirmed as *P. aeruginosa* by API20NE. All isolates were grown with and without $\frac{1}{4}$ MIC of imipenem to act as an inducer. Crude cell-free lysates of these isolates showed potent β -lactamase activity with nitrocephin as reporter substrate, but no imipenem hydrolysis was detected spectrophotometrically. The β -lactamases of these strains failed to focus by IEF, but smeared along the gel from pI 7 to 9, and may be caused by the production of a stably de-repressed chromosomal β -lactamase. Since no imipenem hydrolysis could be detected spectrophotometrically, no further work was conducted on these strains other than the determination of their antibiograms. The antibiograms of these strains are shown in Table 4.2 overleaf.

The remaining strain received from the Glasgow Royal Infirmary was identified as *Enterobacter aerogenes* by API20E. This isolate was resistant to imipenem with an MIC of 32mg/l, above the breakpoint of 16mg/l.

A sonicated cell free lysate of this isolate showed potent nitrocephin hydrolysis, which was resistant to clavulanic acid as shown in Table 4.1. However, no imipenem hydrolysis could be detected spectrophotometrically. Analysis by IEF showed a single smeared band between pI 8 and 9. These data suggest that this strain hyper-produced a β -lactamase possibly as a de-repressed mutant. The biochemical analysis of this enzyme suggested that it may be an Ambler Class C enzyme, possibly similar to the p99 β -lactamase which is common amongst members of the *Enterobacteriaceae*. The antibiograms of these strains are shown in Table 4.2 overleaf.

Table 4.1: Effect of Clavulanic Acid on *Ent. aerogenes* β -lactamase.

Strain	NCFN Hydrolysis ($\mu\text{mol}/\text{min}/\text{ml}$)	NCFN Hydrolysis + Clav ($\mu\text{mol}/\text{min}/\text{ml}$)	% Activity
<i>Ent. aerogenes</i>	1270	1130	89

NCFN- Nitrocephin

Clav - 100 μM Clavulanic Acid.

Table 4.2. MIC Ranges of Isolates Received From Glasgow Royal Infirmary.

Organism	Number of Isolates	Range of Minimum Inhibitory Concentrations (MIC) (mg/l)										
		AMP	AMOX	AMOX / CLAV	CLD	CFX	CTX	CZD	CEFEP	IMP	MERO	CIP
Breakpoint	-	32	32	32	-	32	64	32	32 ^j	16	-	4
<i>P. aeruginosa</i>	4	256->256	>256	256->256	>256	>256	32->256	2-64	4-16	4-32	8-64	0.25-0.5
<i>Ent. aerogenes</i>	1	>256	>256	256	>256	>256	256	256	32	32	16	16

AMP - ampicillin
AMOX - amoxycillin
AMOX/CLAV - amoxycillin / clavulanic acid
CLD - cephaloridine

CFX - cefuroxime
CTX - cefotaxime
CZD - ceftazidime
CEFEF - cefepime

IMP - imipenem
MERO - meropenem
CIP - ciprofloxacin

Boxes shown in grey denote strains which were received as a result of reduced sensitivity to imipenem. For this reason, these strains cannot be regarded as random isolates. As a result of this, these strains may exhibit elevated resistance to several β -lactam antimicrobials, including imipenem.

Breakpoints described by [176].
‡ Cefepime breakpoint proposed by [213].

MIC₅₀ and MIC₉₀ values have not been determined where the number of isolates is less than 9 ($n < 9$).

4.2.1.1. Investigation of the Stability of β -lactamase Production by the *Enterobacter aerogenes* Isolate.

Experiments were conducted to determine the stability of the β -lactamase production by this isolate. To this end, this strain (designated JMS-335) was inoculated into 4.5 ml fresh Nutrient Broth No.2 without any selective pressure every day for 26 days. After this time, the MIC's of both the passaged isolate (designated JMS-335_p), and the original strain (JMS-335) were determined and compared. The MIC's of imipenem of JMS-335 and JMS-335_p were 32mg/l and 8mg/l respectively. This suggested that the hyper-production of the β -lactamase by this isolate was very stable. This reduction in imipenem MIC does result in the strain showing only intermediate resistance to imipenem as defined by NCCLS breakpoint (intermediate - 8mg/l), but this strain remained resistant as defined by BSAC standards (resistant - 8mg/l).

4.2.2. Isolates from Western General Hospital, Edinburgh.

A total of 33 clinical isolates were received from the Western General Hospital of Edinburgh, which all showed reduced sensitivity to imipenem by modified Stokes test. The identities of all these isolates were confirmed by API20E or API20NE as appropriate.

All isolates were grown with and without $\frac{1}{4}$ MIC of imipenem to act as an inducer. Sonicated crude cell-free lysates of all these isolates were examined for β -lactamase activity. With the exception of a single *Sten. maltophilia*, all isolates appeared to produce a β -lactamase, since these preparations hydrolysed nitrocephin by spot test. However, of the single *Enterobacter cloacae*, 11 *P. aeruginosa*, 3 *Burk. cepacia*, and 18 *Sten. maltophilia*, only the *Sten. maltophilia* isolates were able to hydrolyse imipenem spectrophotometrically. Only the *Sten. maltophilia* isolates appeared to produce inducible β -lactamase activity. The antibiograms of these strains were determined, and are shown in Table 4.3 overleaf.

Table 4.3: MIC Ranges of Isolates Received From Western General Hospital.

Organism	Number of Isolates	Range of Minimum Inhibitory Concentrations (MIC) (mg/l)										
		AMP	AMOX	AMOX / CLAV	CLD	CFX	CTX	CZD	CEFEP	IMP	MERO	CIP
Breakpoint	-	32	32	32	-	32	64	32	32 ^j	16	-	4
<i>Burk. cepacia</i>	3	>256	>256	128->256	>256	>256	128->256	8-64	32->256	64-256	16-128	16-64
<i>Ent. cloacae</i>	1	>256	>256	256	128	256	256	64	256	2	2	0.125
<i>P. aeruginosa</i>	11	128->256	>256	128->256	>256	>256	8-128	1-128	2-128	4-32	4-32	0.125-2
-	MIC ₅₀	256	256	256	>256	>256	32	4	8	8	8	0.5
-	MIC ₉₀	>256	>256	>256	>256	>256	128	64	64	32	32	2
<i>Sten. maltophilia</i>	18	128->256	32->256	64-256	64-256	64-256	2-256	0.5-128	1-128	8-256	4-256	0.125-8
-	MIC ₅₀	256	256	128	256	256	64	4	16	64	64	1
-	MIC ₉₀	>256	>256	256	>256	>256	256	64	128	256	256	8

AMP
AMOX
AMOX/CLAV
CLD

- ampicillin
- amoxycillin
- amoxycillin / clavulanic acid
- cephaloridine

CFX
CTX
CZD
CEFEP

- cefuroxime
- cefotaxime
- ceftazidime
- cefepime

IMP
MERO
CIP

- imipenem
- meropenem
- ciprofloxacin

Boxes shown in grey denote strains which were received as a result of reduced sensitivity to imipenem. For this reason, these strains cannot be regarded as random isolates. As a result of this, these strains may exhibit elevated resistance to several β -lactam antimicrobials, including imipenem.

Breakpoints described by [176].

† Cefepime breakpoint proposed by [213].

MIC₅₀ and MIC₉₀ values have not been determined where the number of isolates is less than 9 ($n < 9$).

Since several workers have already examined the L1-type imipenem hydrolysing β -lactamases from *Sten. maltophilia*, and no other strains were able to hydrolyse imipenem, no further work was conducted on these isolates apart from their antibiograms.

The three *Burk. cepacia* isolates showed resistance to all antimicrobials tested, with MICs significantly above the breakpoint for all compounds. It should be noted that these strains were also resistant to both carbapenems, despite the fact that no imipenem hydrolysis was detected spectrophotometrically. Not only were these strains resistant to the carbapenems, but ciprofloxacin resistance was also observed. Of the compounds tested, only ceftazidime and ciprofloxacin showed any activity *in vitro*, although it is unlikely that these compounds would be effective *in vivo* since the concentrations of these compounds required for antibacterial activity are generally greater than those physiologically attainable by infusion.

The *P. aeruginosa* isolates showed extensive resistance to all penicillins and β -lactam/ β -lactamase inhibitor combinations, with MIC₉₀ values above the breakpoints of 32mg/l.

Of the cephalosporins tested, cefotaxime and ceftazidime MIC₅₀ values were below their breakpoints. Ceftazidime MIC₉₀s were only 1 dilution above the breakpoint of 32mg/l. This also applied to the carbapenems. Ciprofloxacin showed greatest activity; MIC₉₀ values for the compound were below the breakpoint of 4mg/l.

The strains of *Sten. maltophilia* examined here were found to be resistant to all penicillins; MIC₉₀ values significantly above the breakpoint of 32mg/l. Ceftazidime and ciprofloxacin were the only compounds which showed an MIC₅₀ below the breakpoints of 32mg/l and 4mg/l respectively. The carbapenems imipenem and meropenem showed little activity, both having MIC₅₀ values of 64mg/l, which were well above the breakpoint of 16mg/l.

4.2.3. Isolates Received From Clinical Laboratories, Edinburgh Royal Infirmary.

4.2.3.1. Aerobes.

Nine *Acinetobacter*, 9 *Enterobacter* and 11 *Klebsiella* isolates were received from the Clinical Bacteriology laboratories, Edinburgh Royal Infirmary. These strains were random consecutive isolates, and were examined for the production of an imipenem hydrolysing β -lactamase. Crude cell-free lysates of these isolates showed nitrocephin hydrolysing activity, suggesting the presence of a β -lactamase. However, none of these extracts were able to hydrolyse imipenem spectrophotometrically, despite some of these isolates having imipenem and meropenem MIC's above the breakpoint of 16mg/l.

Several *Acinetobacter* isolates showed resistance to ciprofloxacin and to the carbapenems. The 'fourth' generation cephalosporin cefepime showed very good activity against both *Enterobacter* and *Klebsiella* isolates, with MIC's comparable to the carbapenems and ciprofloxacin. However, this compound did not show such good activity against *Acinetobacter* isolates.

The third generation cephalosporins cefotaxime and ceftazidime showed good activity against the *Klebsiella* isolates; the MIC₉₀ values being lower than the breakpoints of 64mg/l and 32mg/l respectively. These compounds were not as effective against *Enterobacter*, but MIC₅₀ values were still below their breakpoints. *Klebsiella* and *Enterobacter* MIC₉₀ values for both the carbapenems and also ciprofloxacin were below the breakpoints for these agents, demonstrating the efficacy of these agents.

Of the compounds tested, the only compounds to have significant antibacterial activity against all the species examined were the carbapenems, imipenem and meropenem, and the quinolone, ciprofloxacin. Each of these compounds showed MIC₅₀s below their breakpoints, although individual resistant isolates were found.

The antibiograms of these strains are shown in Table 4.4 overleaf.

Table 4.4: MIC Ranges of Aerobic Isolates Received From Clinical Bacteriology, Edinburgh.

Organism	Number of Isolates	Range of Minimum Inhibitory Concentrations (MIC) (mg/l)										
		AMP	AMOX	AMOX / CLAV	CLD	CFX	CTX	CZD	CEFEP	IMP	MERO	CIP
Breakpoint	-	32	32	32	-	32	64	32	32 [‡]	16	-	4
<i>Acinetobacter</i> spp.	9	8-256	16-256	4-128	64->256	16-256	4-128	2-64	2-64	0.125-32	0.125-16	0.5-32
-	MIC ₅₀	128	128	64	256	64	32	16	16	8	4	2
-	MIC ₉₀	256	256	128	>256	256	128	64	64	32	16	32
<i>Enterobacter</i> spp.	9	16->256	64->256	4->256	4->256	4->256	0.125-128	0.25-128	0.06-1	0.25-2	0.06-0.5	0.03-0.5
-	MIC ₅₀	128	128	64	32	32	2	2	0.25	0.5	0.125	0.125
-	MIC ₉₀	256	256	256	256	256	128	128	1	2	0.5	0.5
<i>Klebsiella</i> spp.	11	8->256	8-128	4-128	4->256	2->256	0.06-64	0.25-64	0.06-1	0.25-8	0.03-2	0.03-0.125
-	MIC ₅₀	128	128	32	32	16	0.25	1	0.25	1	0.125	0.06
-	MIC ₉₀	256	256	128	128	256	32	16	1	4	0.5	0.125

AMP
AMOX
AMOX/CLAV
CLD

- ampicillin
- amoxycillin
- amoxycillin / clavulanic acid
- cephaloridine

CFX
CTX
CZD
CEFEP

- cefuroxime
- cefotaxime
- ceftazidime
- cefepime

IMP
MERO
CIP

- imipenem
- meropenem
- ciprofloxacin

All strains in this table were consecutive random isolates.

Breakpoints described by [176].

‡ Cefepime breakpoint proposed by [213].

4.2.3.2. Anaerobes.

In addition to the aerobes which were received from the clinical laboratories of the Edinburgh Royal Infirmary, a survey examining the antimicrobial resistance of clinical isolates from of the *Bacteroides* group of obligate anaerobes was undertaken. Isolates from the clinical laboratories included strains originally isolated from the Edinburgh Royal Infirmary, and Genito-Urinary Medicine clinic. This survey was conducted over three years from 1993 to 1995. A total of 166 isolates were received and examined.

Sonicated cell-free extracts of each of these isolates were prepared and examined for β -lactamase activity by nitrocephin spot test. All but 7 isolates were able to hydrolyse nitrocephin within 60 minutes, indicating that 95.8% of these isolates produced measurable β -lactamase activity. All isolates were tested spectrophotometrically for imipenem hydrolysing activity. However, no imipenem hydrolysis could be detected in any of these preparations.

The MICs of these isolates were determined against a variety of antimicrobial agents. Table 4.5 overleaf shows the MIC ranges of these isolates against the compounds tested.

In addition to the above strains, several *Bacteroides* isolates were received because they exhibited resistance to imipenem. These isolates cannot be considered as random isolates, and have not been included in this section. They are introduced in the text where applicable.

Table 4.5: Relative Resistance of *Bacteroides* Isolates to Various Antimicrobial Agents.

Item	Minimum Inhibitory Concentration (mg/l)									
	AMP	AMOX	AMOX/ CLAV	CLD	CFX	CTX	CZD	CEFEP	IMP	MERO
Breakpoint	4	4	8	-	-	32	-	32 [†]	16	-
MIC Range	0.06->256	0.03->256	0.015-16	0.03->256	0.06->256	0.03-256	0.03->256	0.06->256	0.03-4	0.015-4
% Resistant	75.9	78.3	9.6			20.4		45.8	0.6	
MIC ₅₀	16	16	0.5	32	32	8	16	32	0.125	0.125
MIC ₉₀	128	128	4	128	256	64	64	256	1	1

AMP

- ampicillin

AMOX

- amoxycillin

AMOX/CLAV

- amoxycillin / clavulanic acid

CLD

- cephaloridine

CFX

- cefuroxime

CTX

- cefotaxime

CZD

- ceftazidime

CEFEP

- cefepime

IMP

- imipenem

MERO

- meropenem

Breakpoints described by [176].

† Cefepime breakpoint proposed by [213].

* These strains exclude JMS-219, JMS-221 and ED-262 [see Chapter 5], since these strains were received because of their elevated resistance to imipenem. JMS-121 is also excluded from this list since it was received as a result of high level resistance to several antimicrobials, and hence could not be considered as a random isolate.

Of all the strains tested as part of the survey, none were found to be resistant to metronidazole by modified Stokes Test, with the *Bact. fragilis* type strain NCTC 9343 as a control. *Bact. fragilis* NCTC 9343 showed a zone of inhibition between 20-26 mm on GBA plates with a 5µg metronidazole disc.

The penicillins ampicillin and amoxycillin showed very limited activity against these isolates, with MIC₅₀ values for these compounds above the breakpoint of 4mg/l. Penicillin was not tested since members of the *Bacteroides fragilis* group are generally regarded as resistant to this compound, as described in Chapter 1. The addition of the β -lactamase inhibitor clavulanic acid to the β -lactam amoxycillin potentiated the bactericidal activity of this β -lactam compound. MIC₉₀ values for this β -lactam / β -lactamase inhibitor combination were 4mg/l and were below the breakpoint of 8mg/l.

Cefotaxime proved to be the most effective of the cephalosporins tested, with MIC₅₀ values of 8mg/l, well below the breakpoint of 32mg/l. However, none of the other cephalosporin compounds proved particularly effective with MICs comparable to or greater than the breakpoint.

Both carbapenems, imipenem and meropenem showed good activity against the anaerobes, with MIC₉₀ values of 1mg/l, well below the NCCLS breakpoint of 16mg/l. Interestingly, these MIC₉₀ values were also below the BSAC 'low' breakpoint of 4mg/l.

Table 4.1: Imipenem Concentrations in Passive Diffusion

Bact. Species	Imipenem	
	MIC Determined (mg/l)	Break concentrations (mg/l)
NCCLS	1.0	16.0
BSAC	1.0	4.0

4.3. Passage Experiments on Two *Bact. fragilis* Isolates.

It was decided to attempt to increase the resistance to imipenem of two *Bact. fragilis* isolates by passage experiments in increasing concentrations of imipenem over 5 consecutive days.

The strains chosen were JMS-63 and JMS-71. Both were received from Dr. D.J. Payne, SmithKline Beecham, Betchworth, Surrey (SB designations ED72 and R416 respectively). When originally sent to Dr. Payne, these strains were found to have MICs of imipenem of 1mg/l and 4mg/l respectively. However, the MICs were found to have dropped to 0.125mg/l and 0.25mg/l. The β -lactamase negative *Bact. fragilis* strain NCTC 9343 was also included in these passage experiments.

The imipenem broth MICs of these strains were found to be higher than their respective plate MIC's. To ensure growth, cultures were set up in 20mls Wilkins-Chalgren broth in McCartney bottles with the following imipenem concentrations.

Table 4.6: Imipenem Concentrations in Passage Experiments.

<i>Bact. fragilis</i> Strain	Imipenem		
	MIC Reported (mg/l)	MIC Determined (mg/l)	Broth concentrations (mg/l)
NCTC 9343	-	0.06	0.03-0.5
JMS-63	1	0.125	0.125-2
JMS-71	4	0.25	0.5-8

The passage experiments were conducted as described in Materials and Methods. The broth with the highest imipenem concentration showing bacterial growth was used to inoculate fresh broth with the same range of imipenem concentrations on the following day. However, the culture failed to grow when inoculated into fresh media on the third day. These experiments were repeated with larger inocula, namely 1%, 2% and 5%, but in each case, the 5 day passage could not be completed. The OD₆₀₀ of a stationary phase culture in this broth varied between 0.8 and 1.2 absorbance units, suggesting only limited growth.

As an alternative, these experiments were repeated in a similar volume of BHI broth in McCartney bottles. The broth MIC's of these strains were found to be lower than their corresponding plate MIC's, but once the imipenem concentrations in the McCartneys had been altered accordingly, the culture failed to grow after day 2 in a similar way to that observed with Wilkins-Chalgren broth. An increase in the inocula did not improve this outcome.

Since neither of these broths are nutritionally rich, the passage experiments were repeated in Robertsons Cooked Meat (RCM) medium in McCartney bottles. However, the broth MIC's of all three strains increased by over an order of magnitude in this medium, and bacterial growth was not consistent from tube to tube. This may have been caused by a variation in the mass of cooked meat granules in each bottle affecting the available imipenem concentration in each bottle.

Finally, these experiments were repeated with thioglycollate medium pH 7.1. The broth MIC's of these strains were comparable with those conducted in Wilkins-Chalgren broth, but again, the cultures would fail to grow before the end of the passage. This outcome was not improved by inoculating a fresh broth with a culture from a lower imipenem concentration.

4.4. *Flavobacterium* Isolates from Culture Collections.

Ten clinical isolates from 9 different species of the genus *Flavobacterium* were received from the National Collection of Type Cultures (NCTC) in London. These strains are discussed in greater detail in Chapter 7. The antibiograms of these strains are shown in Table 4.7 overleaf.

Table 4.7: Relative Resistance of *Flavobacterium* Isolates to Various Antimicrobial Agents.

Item	Number of Isolates	Range of Minimum Inhibitory Concentrations (mg/l)										
		AMP	AMOX	AMOX / CLAV	CLD	CFX	CTX	CZD	CEFEP	IMP	MERO	CIP
Breakpoint	-	32	32	32	-	32	64	32	32 ^s	16	-	4
<i>Flavobacterium</i>	10	2-256	8-256	8-64	4-256	2-256	1-256	2-256	0.25-256	0.25-8	0.125-8	0.25-4
-	MIC ₅₀	64	128	16	32	64	32	32	16	8	2	1
-	MIC ₉₀	256	256	32	256	256	64	128	128	16	16	4
AMP	- ampicillin				CFX	- cefuroxime			IMP	- imipenem		
AMOX	- amoxycillin				CTX	- cefotaxime			MERO	- meropenem		
AMOX/CLAV	- amoxycillin / clavulanic acid				CZD	- ceftazidime			CIP	- ciprofloxacin		
CLD	- cephaloridine				CEFEP	- cefepime						

Flavobacterium isolates are in shaded boxes because they cannot be regarded as random isolates. The reason for their isolation and submission to NCTC is unknown.

Breakpoints described by [176].

^j Cefepime breakpoint proposed by [213].

These isolates were resistant to the penicillins with MIC_{50} s above the breakpoint of 32mg/l. The presence of clavulanic acid in co-amoxyclav resulted in a drop in MIC_{90} to 32mg/l making it equal to the breakpoint. The cephalosporins showed limited activity, cefotaxime being the only compound of this group with MIC_{90} comparable to its breakpoint. The carbapenems showed good activity against these organisms. MIC_{90} s of both of these compounds were 16mg/l, comparable to the breakpoint. The lowest MIC_{90} s were observed for ciprofloxacin, which equalled the breakpoint of 4mg/l for this compound.

CHAPTER FIVE

Optimisation of Divalent Cation Concentration in Assays Involving Metallo- β -lactamases.

5.1. Prologue.

Since the first observation by Sabath and Abraham that metallo- β -lactamases require a divalent transition metal cation, usually zinc for activity [164], many workers have simply included a 1mM zinc sulphate supplement to their assay buffers. Little work has been done on the effect of zinc on catalytic activity apart from the observation that the sequestration of this ion from the metallo- β -lactamase by EDTA and other chelators results in enzyme inactivation. This inhibition can be partially restored by the addition of excess zinc sulphate at a later time, although the degree to which activity is restored varies from enzyme to enzyme.

The only work which suggests any investigation into the effects of zinc on these enzymes was made in 1974 by Davies and Abraham who observed that zinc increased the hydrolytic activity of *B. cereus* II against cephalosporin C [122]. In 1993, Felici and co-workers studied several known metallo- β -lactamases. This group assayed *B. cereus* II in buffer containing 0.3mM ZnCl_2 , whereas all other metallo- β -lactamases were assayed in buffer containing 0.1mM ZnCl_2 [103]. No explanations were given for the choice of these supplement concentrations.

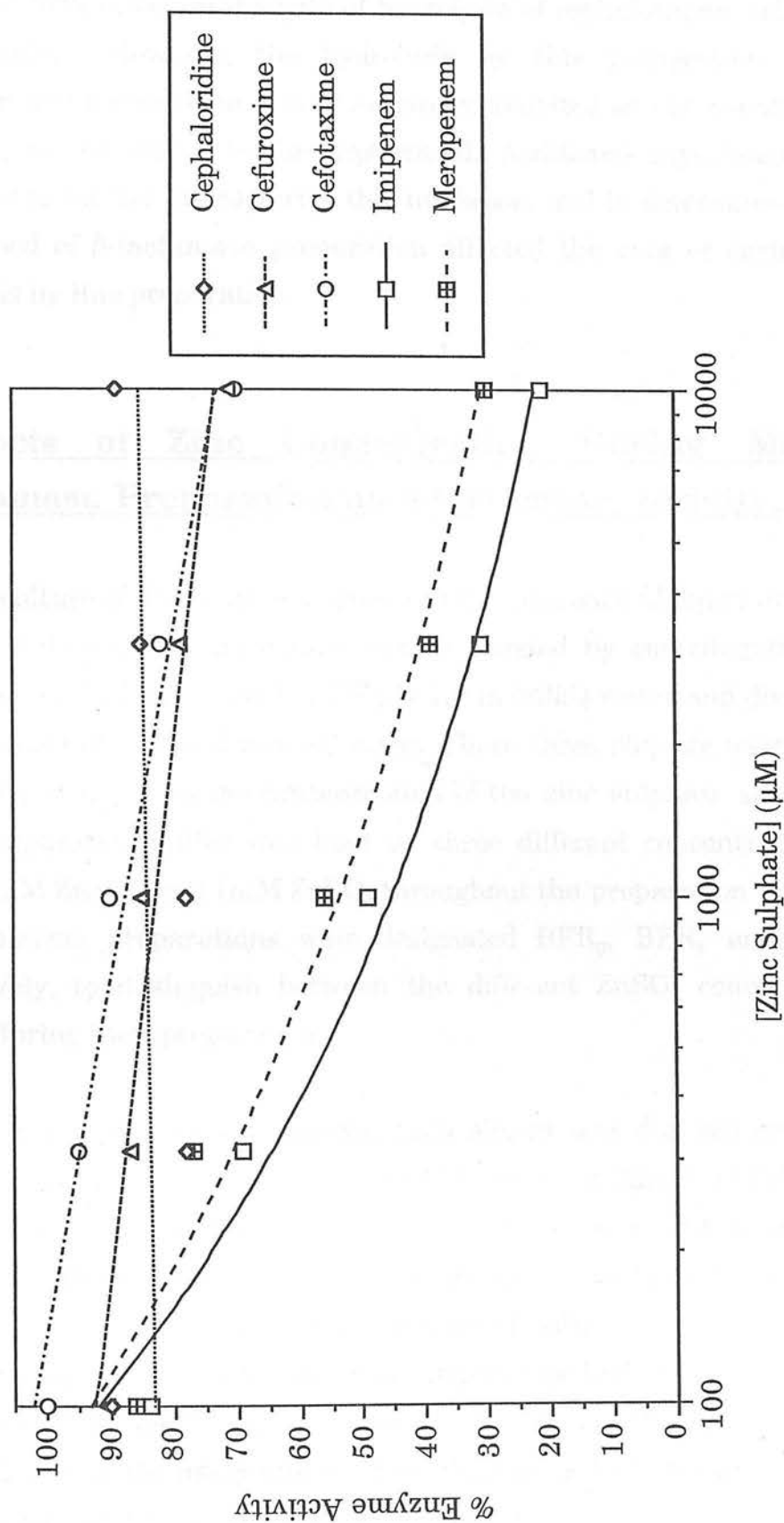
5.2. Effects of Zinc on the Carbapenemase Activity of a Metallo- β -lactamase.

For the assay of these enzymes, an investigation of the effects of zinc on the catalytic activity of these enzymes was undertaken. Experiments were conducted on crude cell free lysates of *Bact. fragilis* JMS-221, which produces a single imipenem hydrolysing β -lactamase, probably a metallo- β -lactamase [see Chapter 6]. Disposable glassware was used wherever possible, and any re-usable glassware was firstly washed in 0.1M EDTA and rinsed in MilliQ water, followed by a wash in 0.1M NaOH and rinsed in MilliQ water until the pH returned to neutral, to ensure that the glass was free of any detergent or excess ions respectively. All bottles had plastic caps, and no rubber inserts were used since it has been reported that such inserts can act as a significant reservoir of metal ions, including zinc [214]. All buffers were prepared in MilliQ water.

A crude sonicated cell-free extract of a 1 litre stationary phase culture was prepared in 25mM PIPES pH 7.0 supplemented with 1mM ZnSO_4 . Prior to any assays, the extract was dialysed four times in a greater than 100-fold excess of 25mM PIPES pH 7.0 in MilliQ water to remove any excess zinc ions. This preparation was then assayed against several substrates in the presence of increasing concentrations of ZnSO_4 . Enzyme activity was plotted as a percentage of basal activity. For the purposes of this thesis, basal activity was taken as the rate of hydrolysis of the tested substrate by the crude preparation in the presence of no ZnSO_4 supplement. Each assay was blanked against substrate with the same zinc concentration as that of the assay mixture.

A graph showing β -lactam hydrolysis against zinc concentration is shown in Figure 5.1 overleaf.

Figure 5.1: Effects of Zinc on Metallo- β -lactamase Hydrolysis of Various Substrates.



It can be seen from Figure 5.1 that the concentration of zinc in the assay buffer had little effect on the rate of hydrolysis of cephaloridine, cefuroxime or cefotaxime. However, the hydrolysis by this preparation of both imipenem and meropenem was increasingly inhibited as the concentration of ZnSO_4 in the assay buffer was raised. Additional experiments were conducted to further characterise this inhibition, and to determine whether the method of β -lactamase preparation affected the rate of carbapenem hydrolysis by this preparation.

5.3. Effects of Zinc Concentration During Metallo- β -lactamase Preparation on β -lactamase Activity.

A 1 litre culture of JMS-121 was grown in the presence of 4mg/l imipenem, and the stationary phase culture was harvested by centrifugation. The pellet was washed in 25mM PIPES pH 7.0 in MilliQ water and divided into three aliquots of identical wet cell mass. These three aliquots were treated identically, except that the concentration of the zinc sulphate supplement in the preparation buffer was kept at three different concentrations; no ZnSO_4 , 1 μM ZnSO_4 , and 1mM ZnSO_4 throughout the preparation procedure. These enzyme preparations were designated BFR_0 , BFR_1 and BFR_{1000} respectively, to distinguish between the different ZnSO_4 concentrations present during their preparation.

Prior to spectrophotometric assays, each aliquot was dialysed separately four times against a greater than 100-fold excess of 25mM PIPES buffer pH 7.0 in MilliQ water to remove any excess zinc ions. The assays were conducted with a variety of reporter substrates known to be hydrolysed by this enzyme. Each enzyme was pre-incubated with varying zinc sulphate concentrations for 5 minutes and then assayed for hydrolytic activity. The basal activity of each aliquot was taken as the rate of β -lactam hydrolysis with no ZnSO_4 in the assay buffer. These data are shown in Figures 5.2, 5.3 and 5.4 overleaf, which are all shown to the same scale to facilitate direct comparisons of these graphs.

Figure 5.2: Effect of Zinc on Metallo- β -lactamase Prepared with no Zinc Sulphate Supplement.

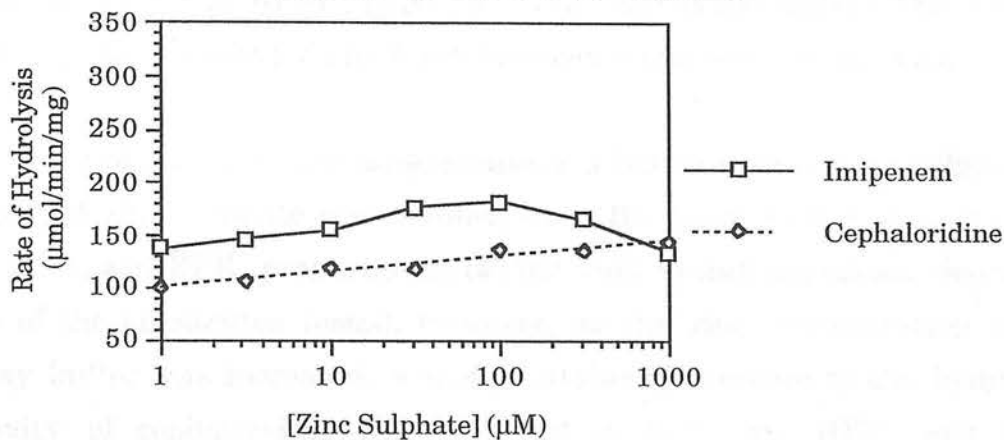


Figure 5.3: Effect of Zinc on Metallo- β -lactamase Prepared with 1 μM Zinc Sulphate Supplement.

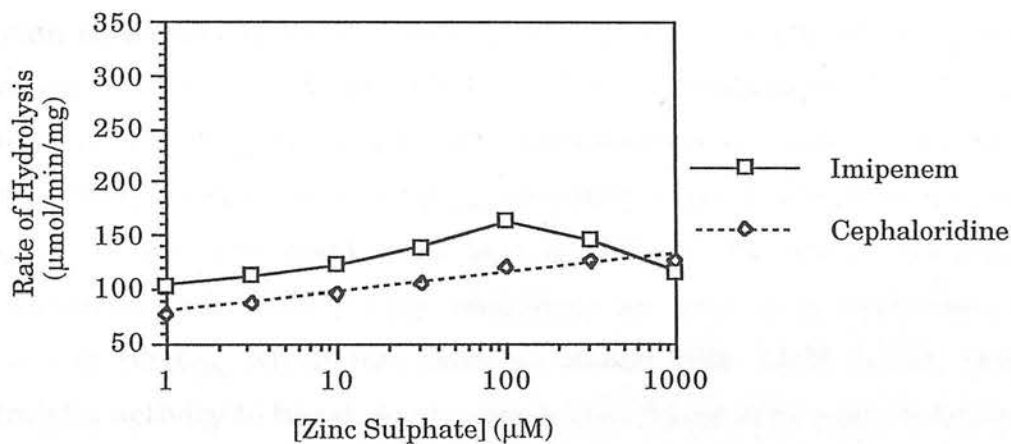
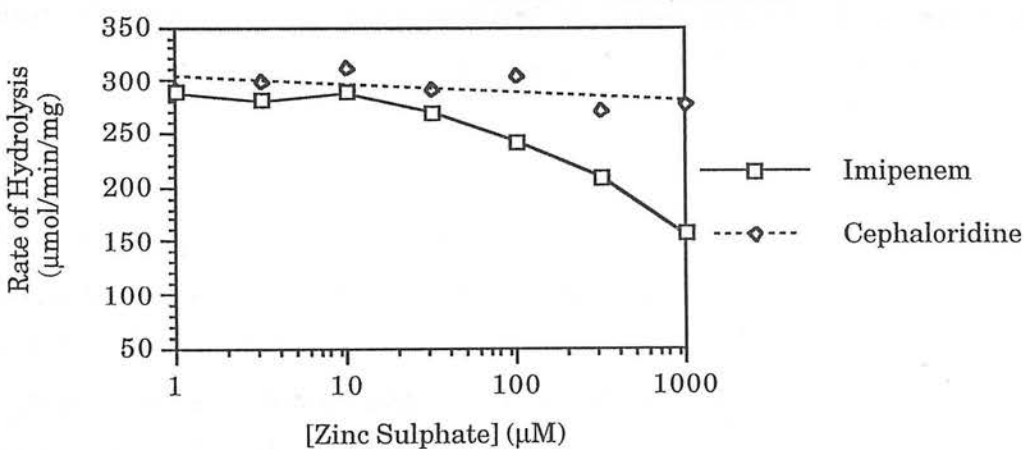


Figure 5.4: Effect of Zinc on Metallo- β -lactamase Prepared with 1 mM Zinc Sulphate Supplement.



Assays were conducted at half log dilutions of zinc sulphate i.e. (1×10^{-3} , 3.16×10^{-4} , 1×10^{-4} , 3.16×10^{-5} , 1×10^{-5} , 3.16×10^{-6} and 1×10^{-6} molar). The value of 3.16 was chosen as it falls half way between 1 and 10 on a \log_{10} plot.

The effect of preparing the β -lactamase in a buffer with no zinc sulphate or with $1 \mu\text{M}$ zinc sulphate was minimal since the basal hydrolytic activity of the BFR_0 and BFR_1 preparations did not vary to any significant degree for any of the substrates tested. However, as the zinc concentration in the assay buffer was increased, a slow, but steady increase in the hydrolytic activity of cephaloridine was observed in both the BFR_0 and BFR_1 preparations.

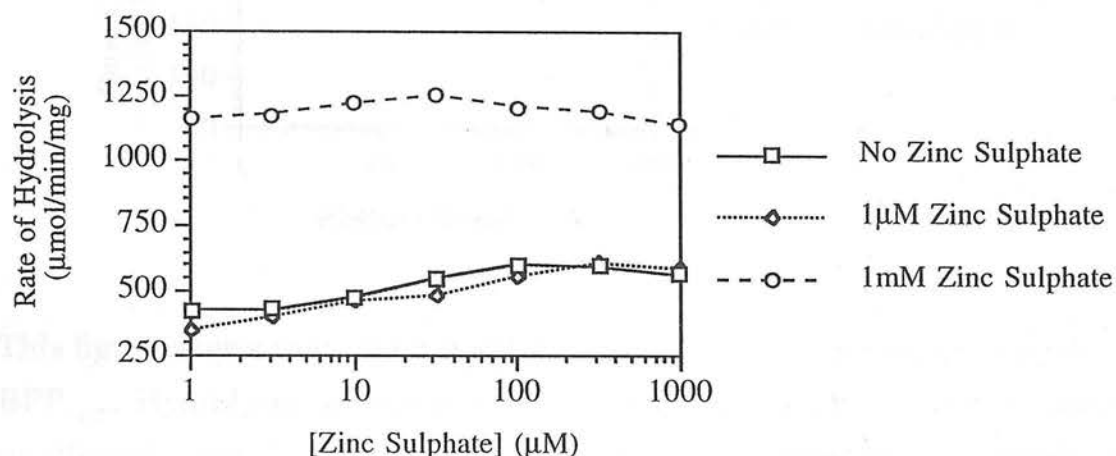
With imipenem as substrate however, BFR_0 and BFR_1 both showed an enzyme *activation* of up to 150% as the ZnSO_4 concentration increased, reaching a peak at $100 \mu\text{M}$ ZnSO_4 for both preparations. The imipenem hydrolysis of BFR_{1000} showed no such activation as the ZnSO_4 concentration was increased. Conversely, BFR_{1000} appeared to be inhibited as this ZnSO_4 supplement was increased, such that an ID_{50} of this compound could be calculated as 1 mM ZnSO_4 . This inhibition was found to be reversible, since dialysis of BFR_{1000} which had been incubated with 1 mM ZnSO_4 restored hydrolytic activity to basal, once a correction for protein concentration had been made.

Whilst the imipenem hydrolysis of BFR_{1000} showed no activation on the addition of ZnSO_4 to the assay mixture, its basal activity was significantly greater than the maximum rate reached by BFR_0 or BFR_1 , even at maximum activation.

It is interesting to note that the rate of hydrolysis of cephaloridine by BFR_{1000} was nearly twice that of BFR_0 and BFR_1 . Cephaloridine hydrolysis by BFR_0 , BFR_1 or BFR_{1000} was unaffected by the ZnSO_4 concentration in the assay buffer. Nitrocephin hydrolysis was also unaffected in this manner, as described in Figure 5.5 overleaf. The nitrocephin hydrolysis of these preparations are shown on a separate graph from Figures 5.2, 5.3 and 5.4 because this substrate is hydrolysed at 3.5 - 4.5 times the rate of

imipenem and cephaloridine [see Chapter 6] and if plotted on the same axes, these effects would not appear as marked.

Figure 5.5: Effects of Zinc During Preparation and Assays on Metallo- β -lactamase Mediated Nitrocephin Hydrolysis.

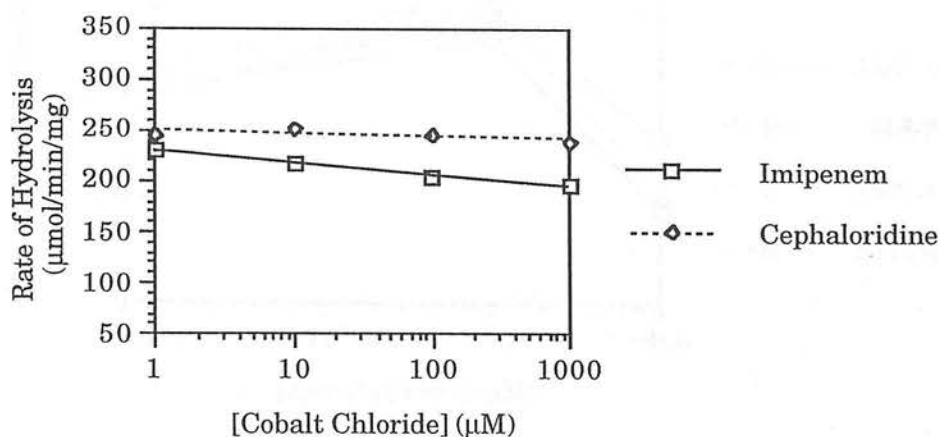


The rate of nitrocephin hydrolysis by BFR₀, BFR₁ or BFR₁₀₀₀ at any zinc sulphate concentration showed no significant difference from basal activity.

5.4. Effects of Cobalt Concentration on Metallo- β -lactamase Activity.

Similar experiments to those conducted with ZnSO_4 were conducted with cobalt, in the form of CoCl_2 in the assay solution to determine whether this inhibition could have been caused by heavy metal poisoning of the β -lactamase. Metallo- β -lactamases are known to be able to function with cobalt instead of zinc as part of their active site, as described in Chapter 1. BFR₁₀₀₀ was assayed in the presence of increasing concentrations of CoCl_2 . These data can be seen in Figure 5.6. Only a slight inhibition of imipenemase activity could be detected at 1mM CoCl_2 . As found with ZnSO_4 , no inhibition of cephaloridine, nitrocephin or cefuroxime hydrolysis was detected. For clarity, only cephaloridine and imipenem are shown in the graph.

Figure 5.6: Effect of Cobalt on Metallo- β -lactamase Prepared with a 1mM Zinc Sulphate Supplement.



This figure shows that cobalt had little effect on the imipenem hydrolysis of BFR₁₀₀₀. Hydrolysis of cephaloridine by this preparation was completely unaffected, and hence implies that the inhibition of imipenem hydrolysis by 1mM ZnSO₄ did not result from heavy metal poisoning.

5.5. Effect of Zinc and Cobalt on Other Metallo- β -lactamases.

5.5.1. *Bact. fragilis* Metallo- β -lactamases.

These experiments were repeated with the metallo- β -lactamases described in Chapter 6 and the known CfiA-type metallo- β -lactamase from *Bact. fragilis* ED-262.

Figure 5.7: Effects of Zinc on Imipenem Hydrolysis of *Bact. fragilis* Metallo- β -lactamases Prepared with no Zinc Sulphate Supplement.

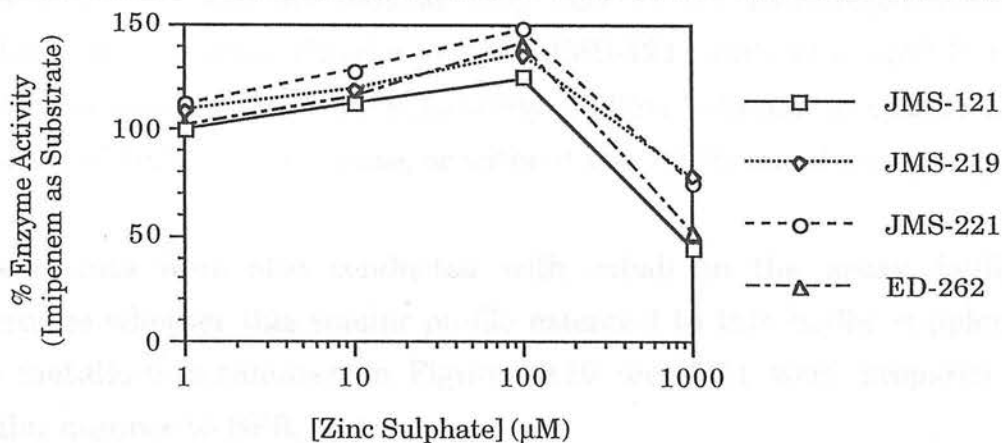


Figure 5.8: Effects of Zinc on Imipenem Hydrolysis of *Bact. fragilis* Metallo- β -lactamases Prepared with a 1mM Zinc Sulphate Supplement.

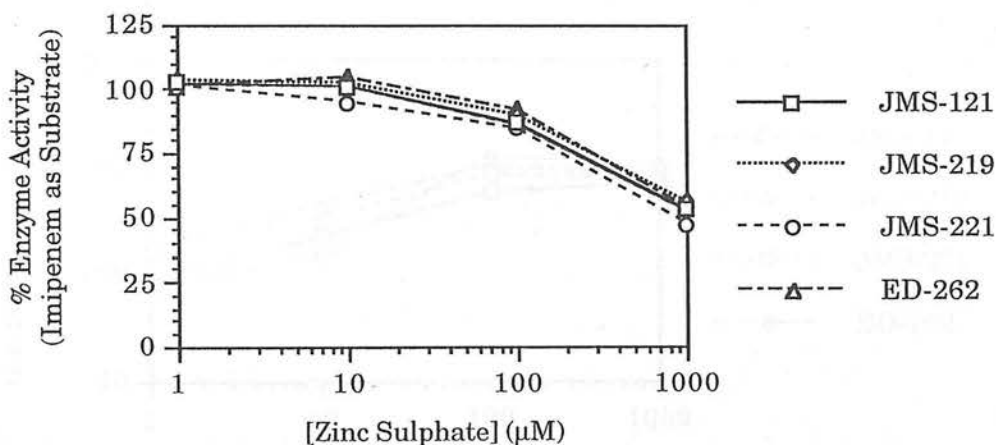
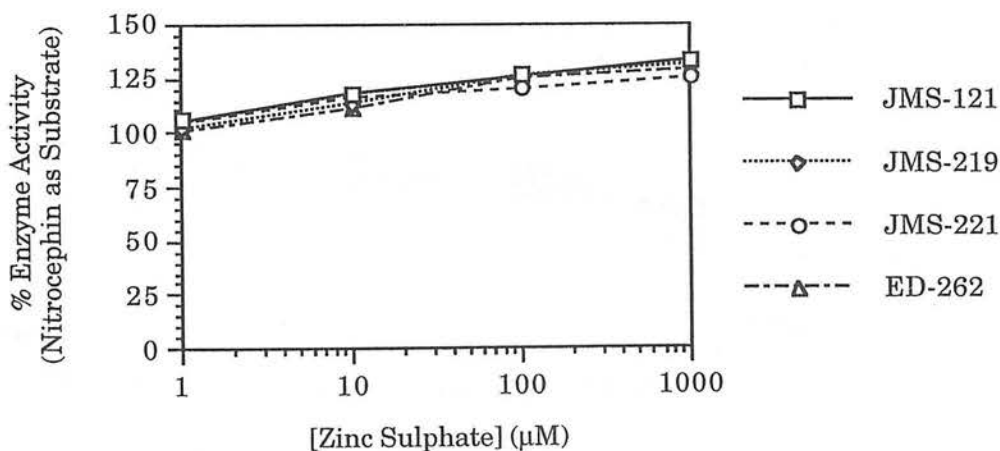


Figure 5.9: Effects of Zinc on Nitrocephin Hydrolysis of *Bact. fragilis* Metallo- β -lactamases Prepared with no Zinc Sulphate Supplement.



Figures 5.7, 5.8 and 5.9 indicate that each of the metallo- β -lactamases produced by the *Bact. fragilis* isolates JMS-121, JMS-219, JMS-221 and ED-262 showed similar zinc sensitivity profiles, whether prepared in the presence of 1mM zinc sulphate, or without this buffer supplement.

Experiments were also conducted with cobalt in the assay buffer to determine whether this similar profile extended to this buffer supplement. The metallo- β -lactamases in Figures 5.10 and 5.11 were prepared in a similar manner to BFR₁₀₀₀.

Figure 5.10: Effect of Cobalt on *Bact. fragilis* Metallo- β -lactamases Prepared with no Zinc Sulphate Supplement.

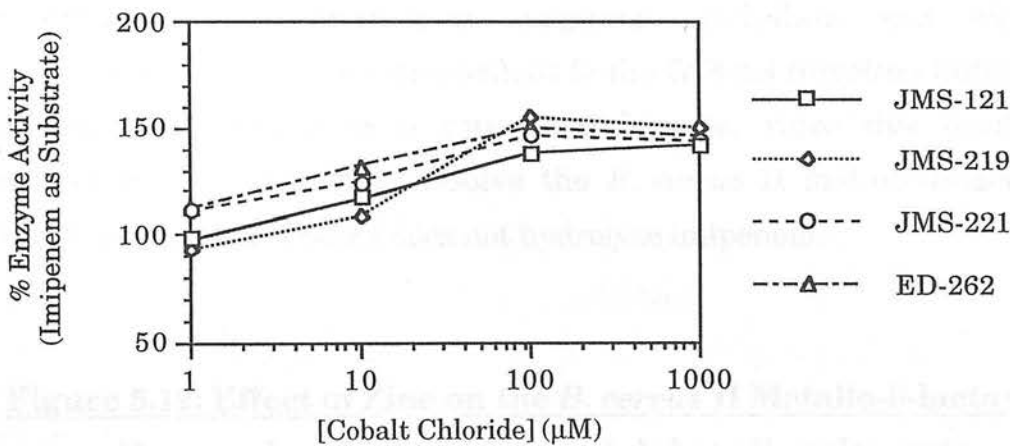
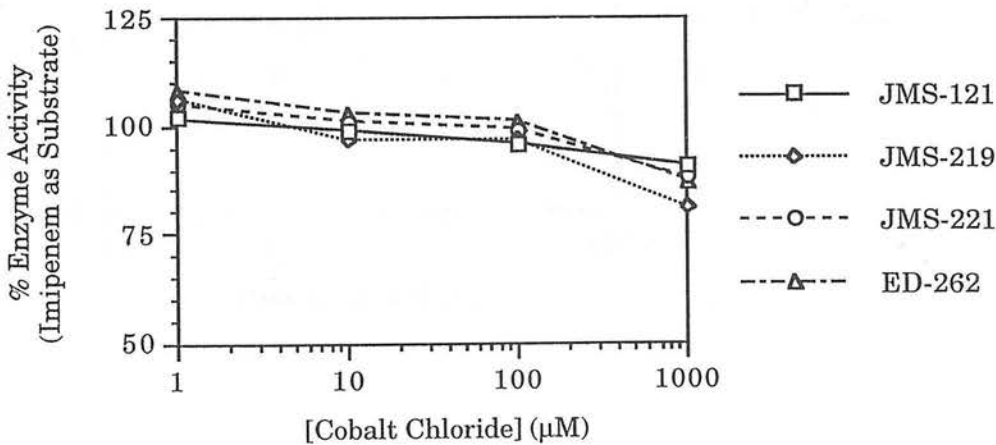


Figure 5.11: Effect of Cobalt on *Bact. fragilis* Metallo- β -lactamases Prepared with a 1mM Zinc Sulphate Supplement.



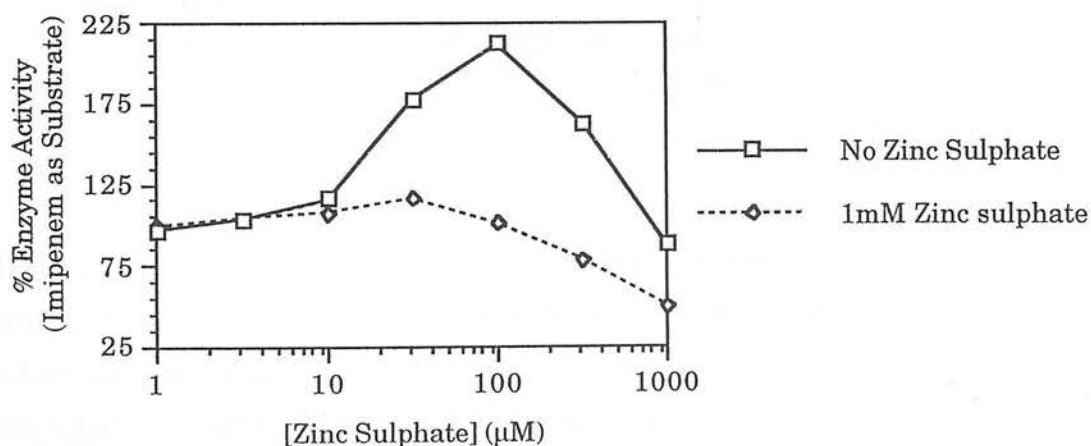
In a similar manner to the zinc data from these enzymes, each of these metallo- β -lactamases demonstrated a similar cobalt sensitivity profile.

5.5.2. *B. cereus* II Metallo- β -lactamase.

These effects appeared to extend to all of the *Bact. fragilis* metallo- β -lactamases examined. Since several other species also produce metallo- β -lactamases, the effects of these ions on the *B. cereus* II metallo- β -lactamase were also investigated.

This enzyme was prepared by selective ammonium sulphate precipitation, followed by Sephadex G75 gel filtration with 25mM PIPES pH 7.0 as the elution buffer. The different zinc sulphate concentrations during enzyme preparation refer to the zinc sulphate concentration in the buffer employed to resuspend the ammonium sulphate precipitate and also the concentration present as a supplement to the G75 gel filtration buffer. Only imipenem was tested as a reporter substrate, since this purification protocol did not completely resolve the *B. cereus* II metallo- β -lactamase from *B. cereus* I. *B. cereus* I does not hydrolyse imipenem.

Figure 5.12: Effect of Zinc on the *B. cereus* II Metallo- β -lactamase Prepared with Various Zinc Sulphate Supplements.

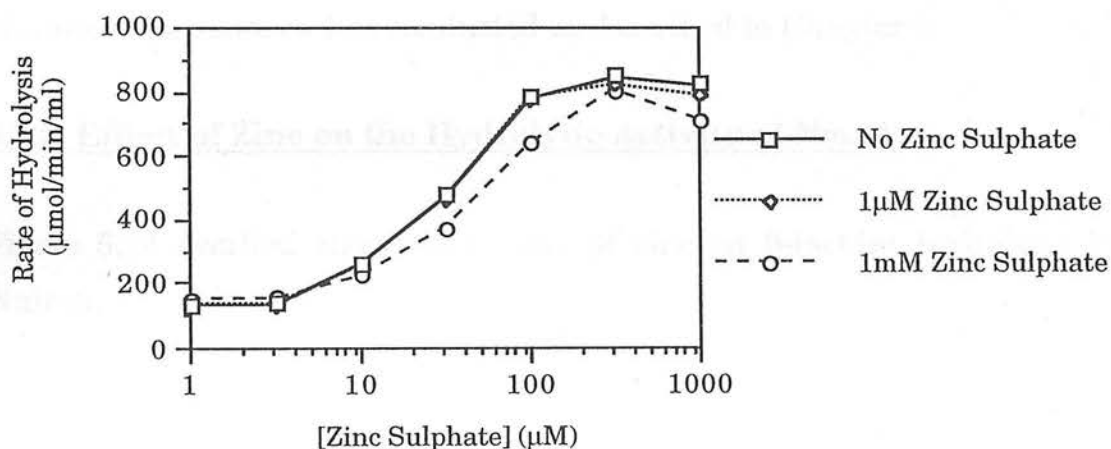


It can be seen from Figure 5.12 that the *B. cereus* II metallo- β -lactamase showed a zinc sensitivity profile similar to that of BFR₁₀₀₀ when prepared in the presence of 1mM ZnSO₄, but when prepared in the absence of a zinc sulphate supplement, this β -lactamase showed greater activation than the *Bact. fragilis* enzymes, and less inhibition at 1mM zinc sulphate.

5.5.3. *Sten. maltophilia* L1.

Crude extracts of the *Sten. maltophilia* L1 metallo- β -lactamase were prepared in a similar manner to BFR₀, BFR₁ and BFR₁₀₀₀. These preparations were designated L1₀, L1₁ and L1₁₀₀₀. Imipenem was the only reporter substrate examined, since this preparation was a crude extract, and the L2 cephalosporinase was still present in the preparation. This latter enzyme does not hydrolyse imipenem.

Figure 5.13: Effect of Zinc on the *Sten. maltophilia* L1 Metallo- β -lactamase Prepared with Various Zinc Sulphate Supplements.



The *Sten. maltophilia* L1 metallo- β -lactamase exhibited a very different zinc sensitivity profile to *B. cereus* II or the *Bact. fragilis* metallo- β -lactamases. The concentration of the zinc sulphate supplements in the preparation buffer did not affect the zinc sensitivity profile of this enzyme since L1₀, L1₁ and L1₁₀₀₀ each showed a similar sigmoidal activation curve, although the degree of activation is slightly less marked in L1₁₀₀₀.

5.6. Effect of Divalent Cations on Serine Active Carbapenemases.

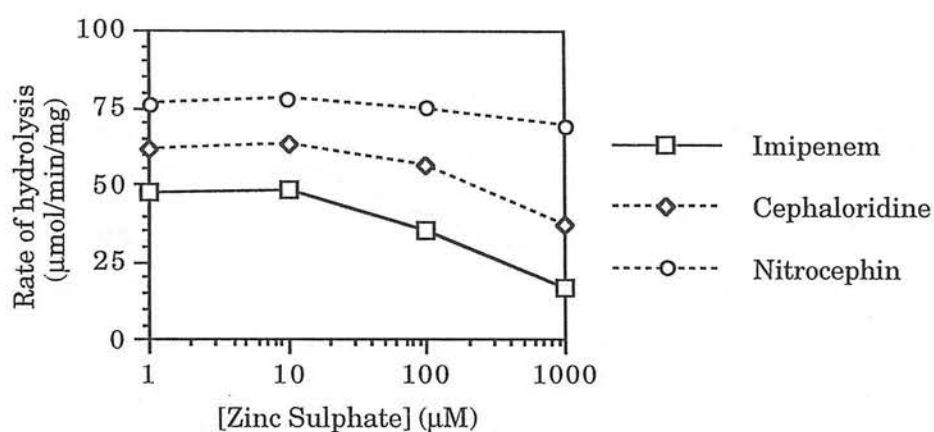
5.6.1. Prologue.

It was postulated that this zinc mediated inhibition of carbapenem hydrolysis may have been caused by the binding of several zinc ions to the active site of a metallo- β -lactamase, since *B. cereus* II has been shown to possess more than one zinc binding site [see Chapter 1].

To test this hypothesis, a β -lactamase with a different mechanism of action was required. Since this phenomenon was restricted to carbapenem hydrolysis, Nmc-A, the only serine active carbapenemase known at that time was examined under similar conditions to see whether this phenomenon was restricted to metallo- β -lactamases, or applied to carbapenem hydrolysis by all carbapenemases. *Enterobacter cloacae* NOR-1 produces two β -lactamases, the serine active site carbapenemase NmcA, and a chromosomal AmpC type cephalosporinase. The separation of these β -lactamases was conducted as described in Chapter 3.

5.6.2. Effect of Zinc on the Hydrolytic Activity of NmcA.

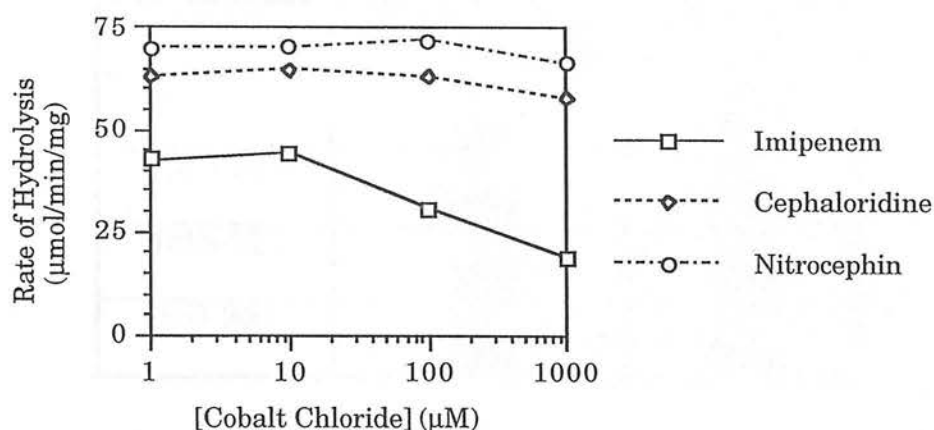
Figure 5.14 overleaf shows the effect of zinc on β -lactam hydrolysis by Nmc-A.

Figure 5.14: Effect of Zinc on the β -lactamase Activity of Nmc-A.

It can be seen from Figure 5.14 that the hydrolysis of both imipenem and cephaloridine were inhibited as the ZnSO_4 concentration was increased. In a similar way to BFR_{1000} , this inhibition was reversed after dialysis. Nitrocephin however, showed no significant inhibition, even at 1mM zinc sulphate.

5.6.3. Effect of Cobalt on the Hydrolytic Activity of NmcA.

Since zinc inhibited the hydrolysis of both imipenem and cephaloridine by Nmc-A, the effect of cobalt on this serine active carbapenemase was also determined. These data are shown in Figure 5.15 overleaf.

Figure 5.15: Effect of Cobalt on the β -lactamase Activity of Nmc-A.

It can be clearly seen from this figure that the addition of cobalt chloride to the assay buffer had no significant effect on the ability of NmcA to hydrolyse cephaloridine or nitrocephin. Interestingly, as the concentration of this ion approached 1mM, significant inhibition of imipenem hydrolysis could be detected. Dialysis of this sample demonstrated that this inhibition was reversible.

5.7. Effect of Zinc on Metallo- β -lactamase Inhibitor Studies.

The above sections state that the presence of zinc during β -lactamase preparation and assays has an effect on hydrolytic activity. The concentration of these supplements also affect ID_{50} values for a diagnostic metallo- β -lactamase inhibitor. The ID_{50} of EDTA is partially dependent upon zinc concentration during β -lactamase preparation, as described in Table 5.1 overleaf.

Table 5.1: Effect of Zinc on Metallo- β -lactamase Inhibition by EDTA.

Strain	[ZnSO ₄] during preparation (μ M)	EDTA ID ₅₀ (μ M)
JMS-121	0	200
	1000	1000
JMS-219	0	250
	1000	1000
JMS-221	0	300
	1000	1000
ED-262	0	150
	1000	1000

It can be seen from Table 5.1 above that the presence of a 1mM zinc sulphate supplement during β -lactamase preparation caused a 3 to 5 fold increase in the apparent ID₅₀ for this compound. This is despite dialysis against buffer without a zinc sulphate supplement prior to ID₅₀ determination.

CHAPTER SIX

Characterisation of the Metallo- β -lactamases from 3 *Bacteroides fragilis* Clinical Isolates.

6.1. Prologue.

In addition to the survey of *Bacteroides* group isolates, several other *Bact. fragilis* clinical isolates were also received for examination. These isolates have not been included in the survey conducted in Chapter 4, since these isolates were received from various sources, and were sent because they exhibited reduced sensitivity to imipenem. It should be noted that all strains examined in this section were originally isolated from geographically distinct regions within Great Britain.

6.2. Case Histories.

Bact. fragilis JMS-121 was received from the Scottish Anaerobe Reference Laboratory, and was originally isolated from a 50 year old man with a duodenal ulcer. This isolate had previously been shown to be resistant to metronidazole [189] but no information about its sensitivity to imipenem was known.

Bact. fragilis JMS-219 was received from the Public Health Service Laboratory (PHLS), Cardiff. This isolate was discovered in 1992 at Ealing Hospital, and was originally isolated from an 82 year old woman with leg and heel blisters. This strain was also reported to be resistant to metronidazole (Dr. J. Brazier, Pers. Comm.).

Bact. fragilis JMS-221 was also received from the PHLS, Cardiff. This clinical isolate was discovered at Ninewells Hospital, Dundee, and was originally isolated from the abdominal wounds of a patient with myelofibrosis (Dr. J. Brazier, Pers. Comm.).

6.3. Initial Studies.

1. *Bact. fragilis* strains JMS-121, JMS-219 and JMS-221 were all clinical isolates, and were found to be resistant to imipenem and meropenem by breakpoint.
2. Sonicated crude cell-free lysates of each of these isolates were found to be able to hydrolyse imipenem spectrophotometrically.
3. Incubation of these extracts with 10mM EDTA overnight caused a greater than 99% reduction in both imipenem and nitrocephin hydrolysis for all three isolates. This incubation had no effect on the nitrocephin hydrolysis of the TEM-1 β -lactamase.
4. A five minute incubation of the EDTA dialysed samples with 1mM zinc sulphate resulted in restoration of ~25% of the nitrocephin hydrolysing activity and 20-26% of imipenem hydrolysing activity. These data are described in Table 6.7.

For these reasons, all three isolates were chosen for further study. All three isolates were confirmed as *Bact. fragilis* by API32A.

MIC data were determined for these isolates, against a variety of antimicrobial agents. Table 6.1 overleaf details the antibiograms of these isolates.

Table 6.1: Minimum Inhibitory Concentrations of Imipenem Resistant *Bact. fragilis* Isolates.

Strain	Minimum Inhibitory Concentration (mg/l)										
	AMP	AMOX	AMOX / CLAV	CLD	CFX	CTX	CZD	CEFEP	MERO	IMP	IMP / BRL
Breakpoint	32	32	32	-	32	64	32	32 [‡]	-	16	-
JMS-121	>256	>256	64	256	>256	>256	>256	>256	>256	>256	256
JMS-219	>256	256	32	256	>256	>256	>256	>256	16	16	8
JMS-221	>256	>256	64	>256	>256	>256	>256	>256	256	256	128
NCTC 9343	32	32	2	32	8	4	4	16	0.03	0.06	<0.03

AMP
AMOX
AMOX/CLAV
CLD
CFX
IMP/BRL (2:1)
IMP/BRL 2
NCTC 9343

- ampicillin
- amoxycillin
- amoxycillin / clavulanic acid
- cephaloridine
- cefuroxime
imipenem / BRL42715 in a 2:1 ratio.
imipenem / BRL42715 (BRL42715 at 2mg/l)
β-lactamase negative *Bact. fragilis* type strain included for control purposes.

CTX
CZD
CEFEP
IMP
MERO

- cefotaxime
- ceftazidime
- cefepime
- imipenem
- meropenem

Breakpoints described by [176].
‡ Cefepime breakpoint proposed by [213].

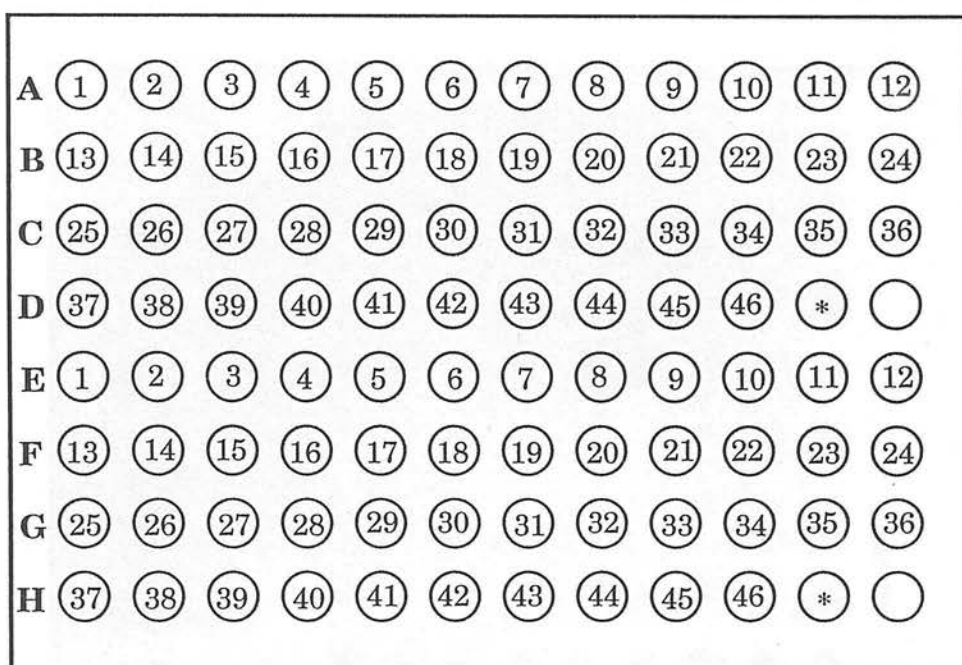
JMS-121 and JMS-221 both produced a doublet band of β -lactamase activity at pI 4.6-5.0 by IEF. JMS-219, however failed to focus by this method. Gel filtration and iso-electric focusing suggested that these strains each produced a single β -lactamase since column fractions showed a single peak of β -lactamase activity, with a constant ratio of nitrocephin:imipenem hydrolysis, and active fractions co-focused at pI 4.6-5.0 with crude cell-free lysates.

This was supported by the observation that tazobactam had little inhibitory effect on the rate of substrate hydrolysis. This indicated that no significant hydrolysis was mediated by another β -lactamase as the previously described [215] *Bact. fragilis* chromosomal β -lactamase (Bush classification group 2e) was found to be sensitive to tazobactam [see also Section 1.9.6.4].

6.4. Purification of Imipenemases from *Bact. fragilis* Clinical Isolates by FPLC.

To determine the best ion exchange matrix to purify these enzymes, crude cell-free extracts of the isolates were tested against 46 different matrices as described in Materials and Methods. A diagrammatic view of the microtitre plate layout is shown in Figure 6.1 overleaf. A photograph of the actual microtitre plates showing matrix binding is shown in Figure 6.2 and Figure 6.3.

The binding of a β -lactamase to a specific matrix was detected by the addition of the chromogenic cephalosporin nitrocephin to the matrix supernatant. Matrices which bound β -lactamase activity remained yellow, whilst unbound β -lactamase remained in the supernatant and hydrolysed the yellow nitrocephin to a red product.

Figure 6.1: Diagrammatic View of Microtitre Assay Plate.

The number within the microtitre well denotes the matrix in that particular well. Since each set of 46 matrices only filled four rows of the plate, it was possible to assay up to two samples per microtitre plate. Where applicable, Rows E-H were loaded with matrix in a similar manner to rows A-D, to facilitate the assay of another enzyme.

Figure 6.2: Microtitre Plate Showing the Relative Binding of JMS-121 and JMS-219 β -lactamase to 46 Ion Exchange Matrices.

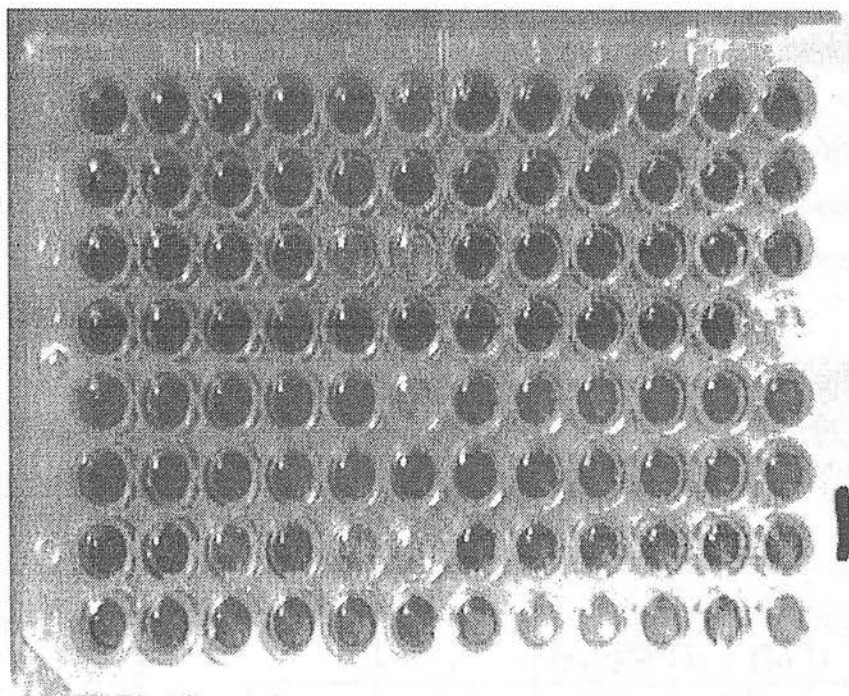
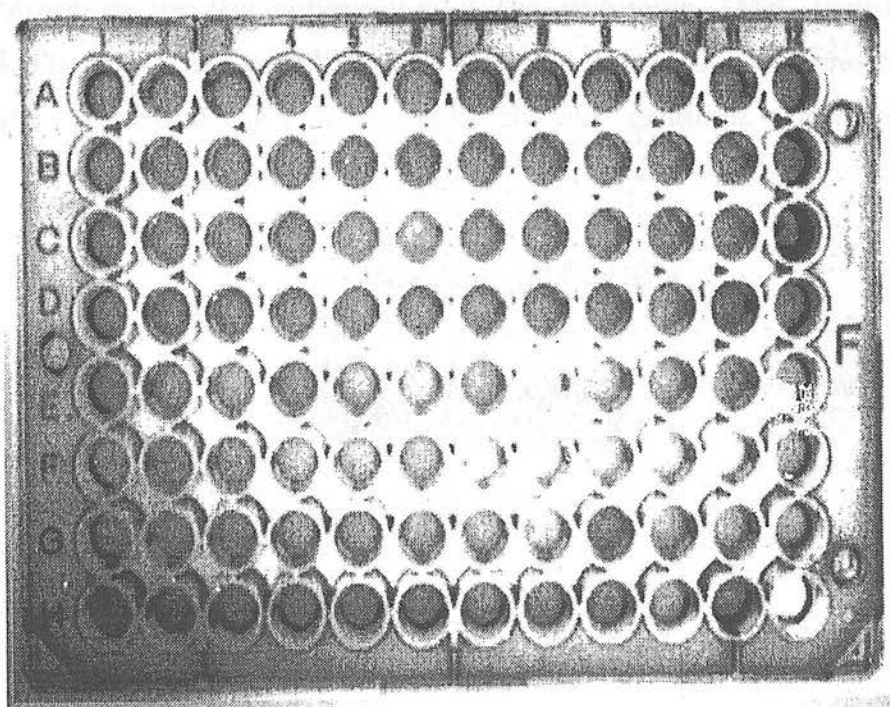


Figure 6.3: Microtitre Plate Showing the Relative Binding of the β -lactamase from JMS-221 to 46 Ion Exchange Matrices.



From Figures 6.2 and 6.3, it was clear that matrix 30 (MacroPrep High Q) showed greatest β -lactamase binding. It should also be noted that matrix 6 (ω -Amino Hexyl Sepharose) and matrix 29 (Q-Sepharose) also showed some binding. This binding profile was similar for all three enzymes, and so a MacroPrep High Q column in 20mM Bis Tris buffer pH 6.0 with 0.1mM ZnSO_4 was used to purify these enzymes. *Bact. fragilis* ED-262, a strain known to be a CfiA metallo- β -lactamase producer was also purified on this column under similar buffer conditions. Table 6.2 below shows the FPLC parameters for each strain.

Table 6.2: FPLC Parameters from Separation of *Bact. fragilis* β -lactamases.

Strain	Column matrix volume (ml)	Flow rate (ml/min)	NaCl Gradient (ml)		Total Run volume (ml)
			0-0.5M	0.5-1M	
JMS-121	58	2.0	175	85	260
JMS-219	58	2.0	175	85	260
JMS-221	58	2.0	175	85	260
ED-262	48	2.0	100	100	200

The parameters for the separation of the unknown JMS- isolates were identical. The protocol employed a linear salt gradient between no NaCl and 0.5M NaCl for 67% of the run time, and the salt gradient was altered such that the remaining 0.5-1M NaCl gradient would be completed in the remaining 33% of the run-time.

However, the parameters were altered for the purification of the ED-262 (known CfiA-type) β -lactamase purely to reduce the column run-time, and hence run volume. The column was run at the same rate, but since the total run volume had been reduced, a linear NaCl gradient between 0M and 1M was employed.

Figures 6.4-6.7 overleaf shows the purification chart of these four strains by FPLC.

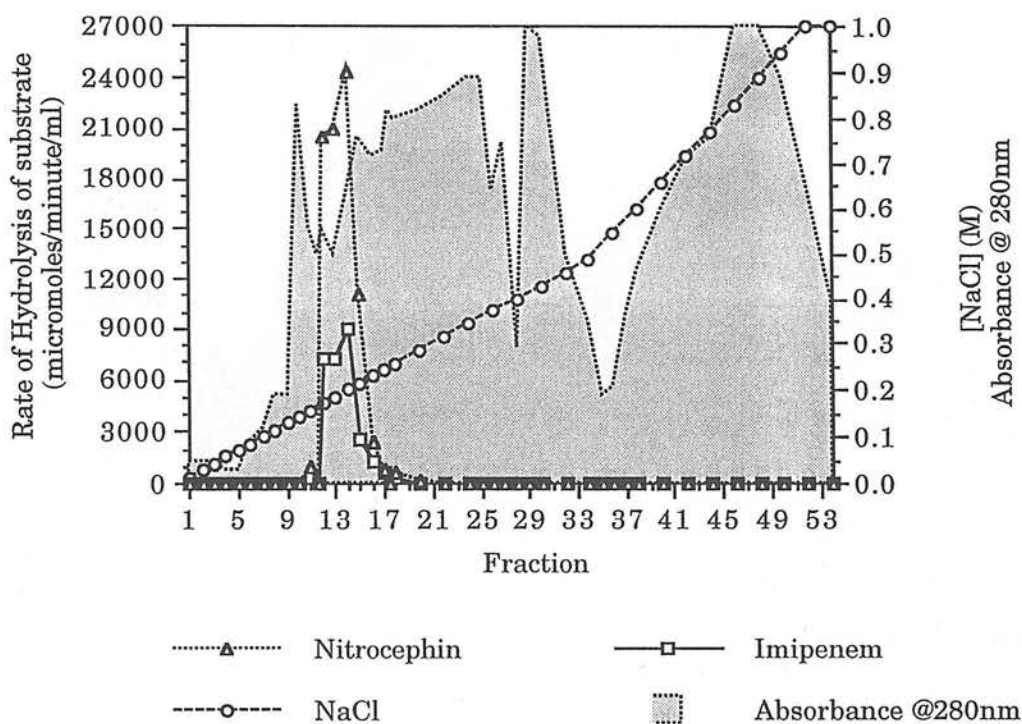
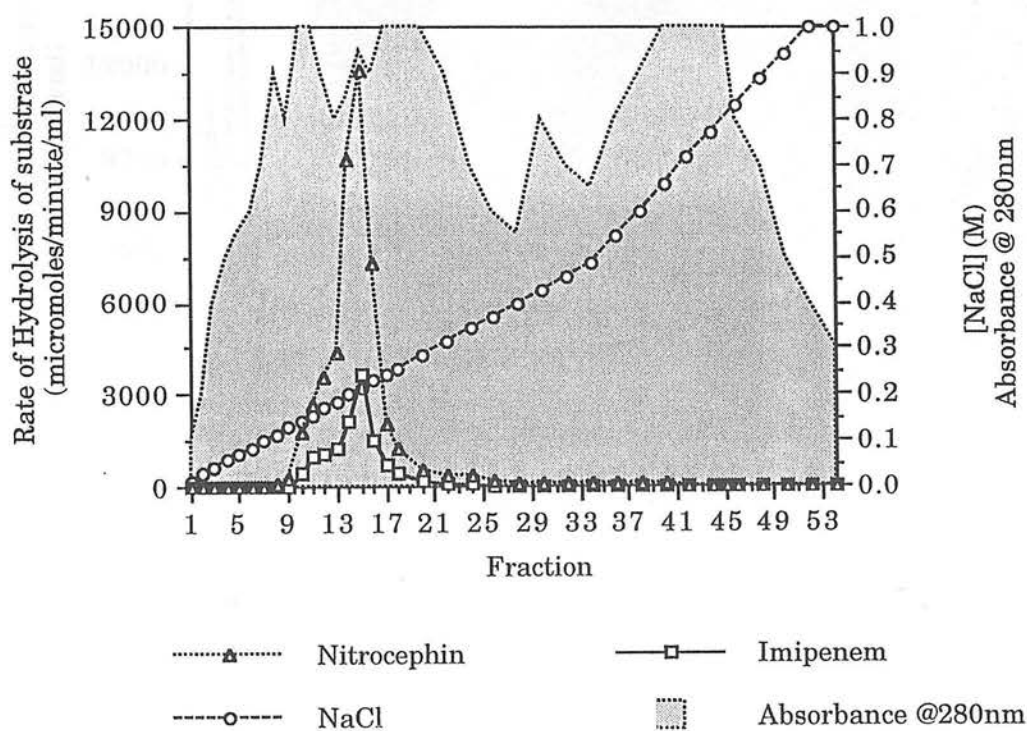
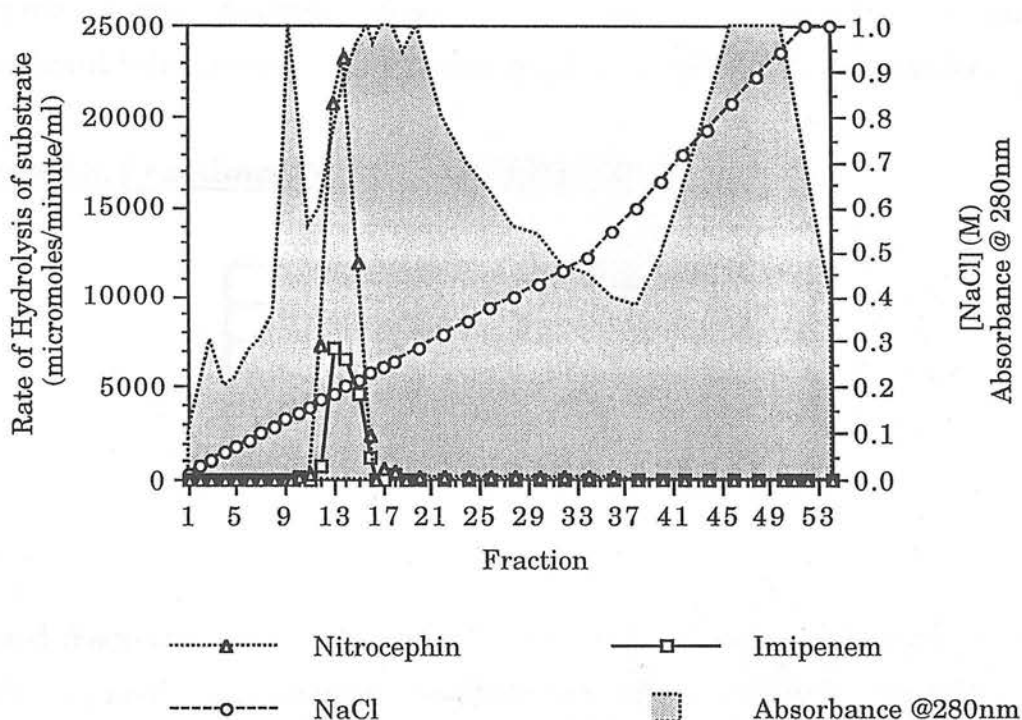
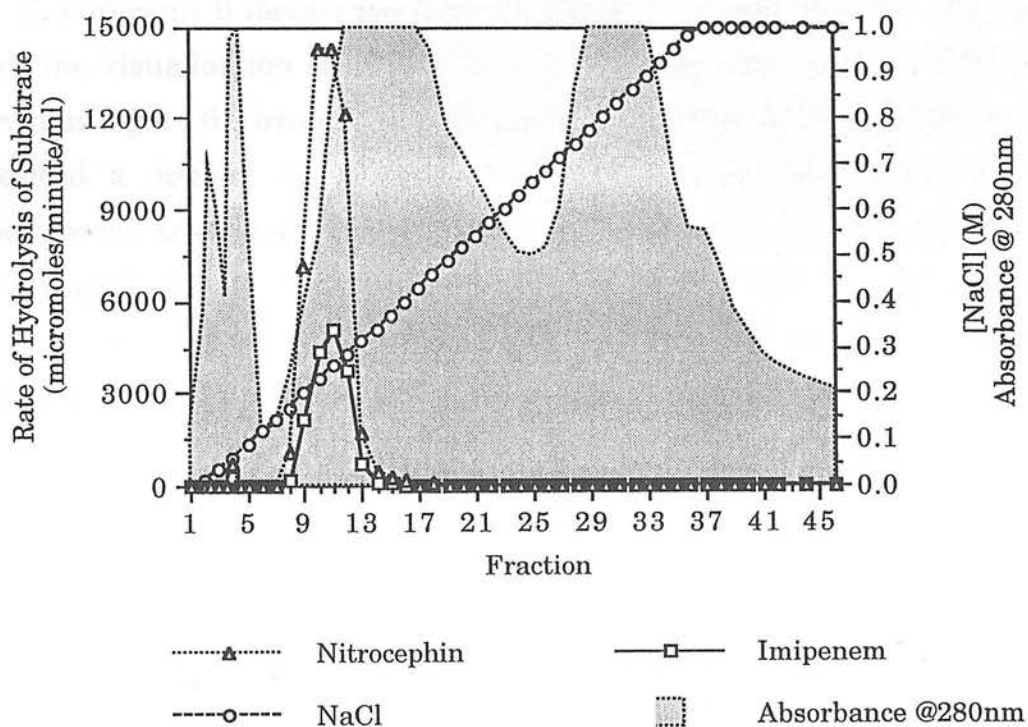
Figure 6.4: FPLC Purification Trace from *Bact. fragilis* JMS-121.**Figure 6.5: FPLC Purification Trace from *Bact. fragilis* JMS-219.**

Figure 6.6: FPLC Purification Trace from *Bact. fragilis* JMS-221.**Figure 6.7: FPLC Purification Trace from *Bact. fragilis* ED-262.**

It can be seen from Figures 6.4, 6.5, 6.6 and 6.7 that each of these enzymes exhibited similar elution profiles from the MacroPrep High Q column; each enzyme eluting between fractions 10 and 16. Fractions containing significant β -lactamase activity were pooled, as described in Table 6.3.

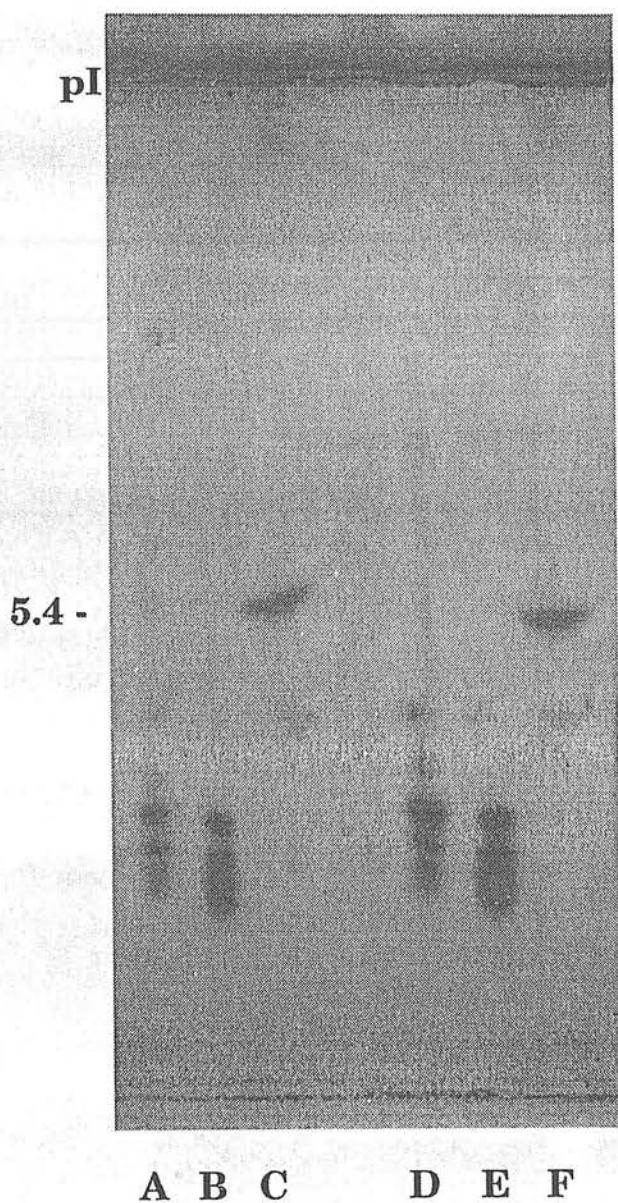
Table 6.3: Fractions Pooled after FPLC Purification.

Strain	Fractions Pooled
JMS-121	12-16
JMS-219	12-16
JMS-221	12-16
ED-262	10-13

Pooled fractions were dispensed into 1ml aliquots and maintained at -20°C until required. Biochemical analysis of FPLC purified enzymes was conducted within one week of purification wherever possible.

Whilst crude extracts of JMS-219 failed to focus by IEF, once purified by FPLC, sufficient β -lactamase activity could be loaded onto the IEF gel to allow the visualisation of all four β -lactamases by this method. This gel is shown in Figure 6.8 overleaf, and it can be seen that all three JMS- strains produced a doublet at pI 4.6-5.0 which is characteristic of the CfiA β -lactamase from both the original TAL 2480 *Bact. fragilis* isolate, and *Bact. fragilis* ED-262 which is known to produce a CfiA-type β -lactamase. This adds further credence to the suggestion that all three of these JMS- strains produced a CfiA related β -lactamase.

Figure 6.8: Iso-electric Focusing Gel of β -lactamases from *Bact. fragilis* Isolates and the known CfiA Producer ED-262.



Lane A	β -lactamase from JMS-121.
Lane B	β -lactamase from JMS-219.
Lane C	TEM-1.
Lane D	β -lactamase from JMS-221.
Lane E	β -lactamase from ED-262.
Lane F	TEM-1.

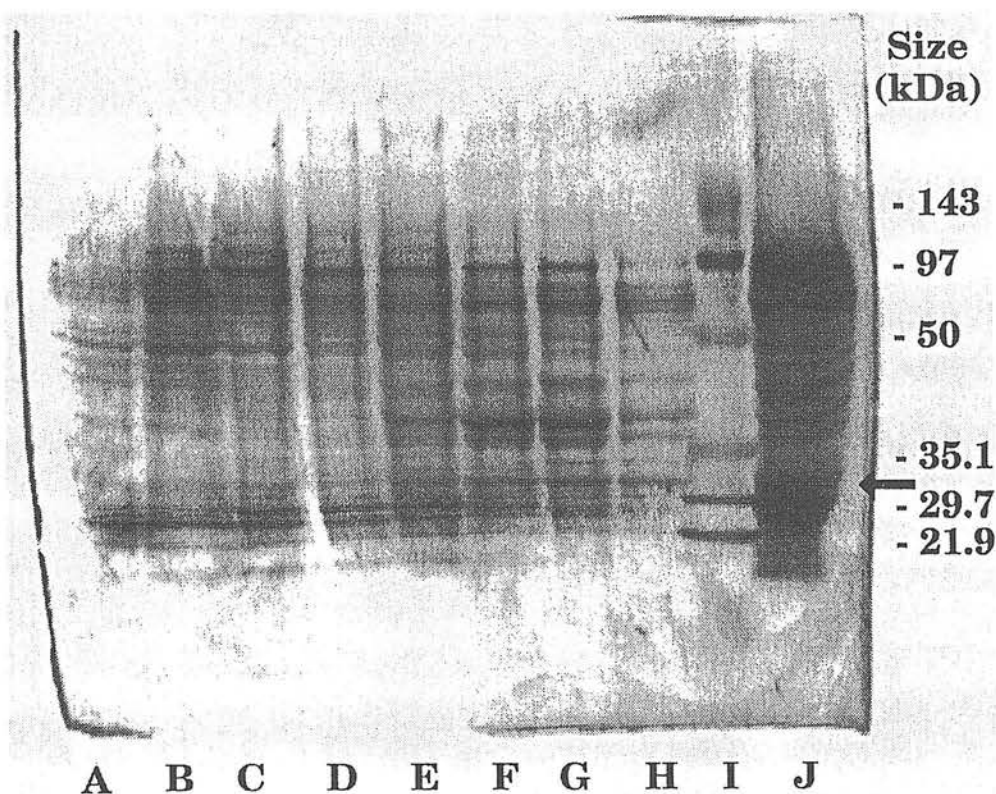
SDS-PAGE of the FPLC fractions of the JMS- enzymes indicate that all these proteins were of approximately 31kDa which correlated with previously published values [see Table 1.4] Table 6.4 below compares the apparent molecular weights of these enzymes.

Table 6.4: Molecular Weights of *Bact. fragilis* β -lactamases.

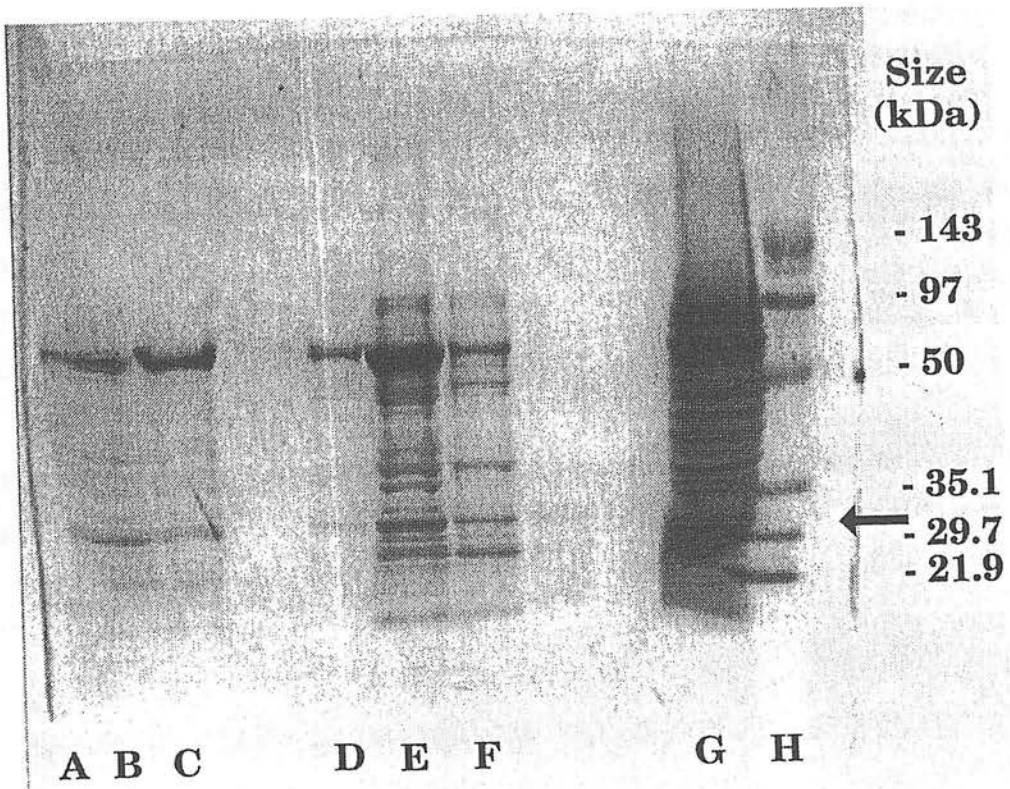
Strain	Molecular weight (kDa)	
	SDS-PAGE	Gel Filtration
JMS-121	31	24
JMS-219	31	21
JMS-221	31	25
ED-262	31	ND
Published Range ^a	26-44	-

ND - Not Determined

^a See Chapter 8 for sources of published range.

Figure 6.9: SDS-PAGE of *Bacteroides* Isolates.

Lane A	JMS-121 FPLC fraction 11.
Lane B	JMS-121 FPLC fraction 12.
Lane C	JMS-121 FPLC fraction 13.
Lane D	JMS-121 FPLC fraction 14.
Lane E	JMS-121 FPLC fraction 15.
Lane F	JMS-121 FPLC fraction 16.
Lane G	JMS-121 FPLC fraction 17.
Lane I	Protein molecular weight markers.
Lane J	Crude JMS-121.

Figure 6.10: SDS-PAGE of *Bacteroides* Isolates.

Lane A	JMS 219 FPLC fraction 12.
Lane B	JMS-219 FPLC fraction 15.
Lane C	JMS-219 FPLC fraction 18.
Lane D	JMS-221 FPLC fraction 12.
Lane E	JMS-221 FPLC fraction 15.
Lane F	JMS-221 FPLC fraction 18.
Lane G	JMS-221 crude cell-free lysate.
Lane H	Molecular weight markers.

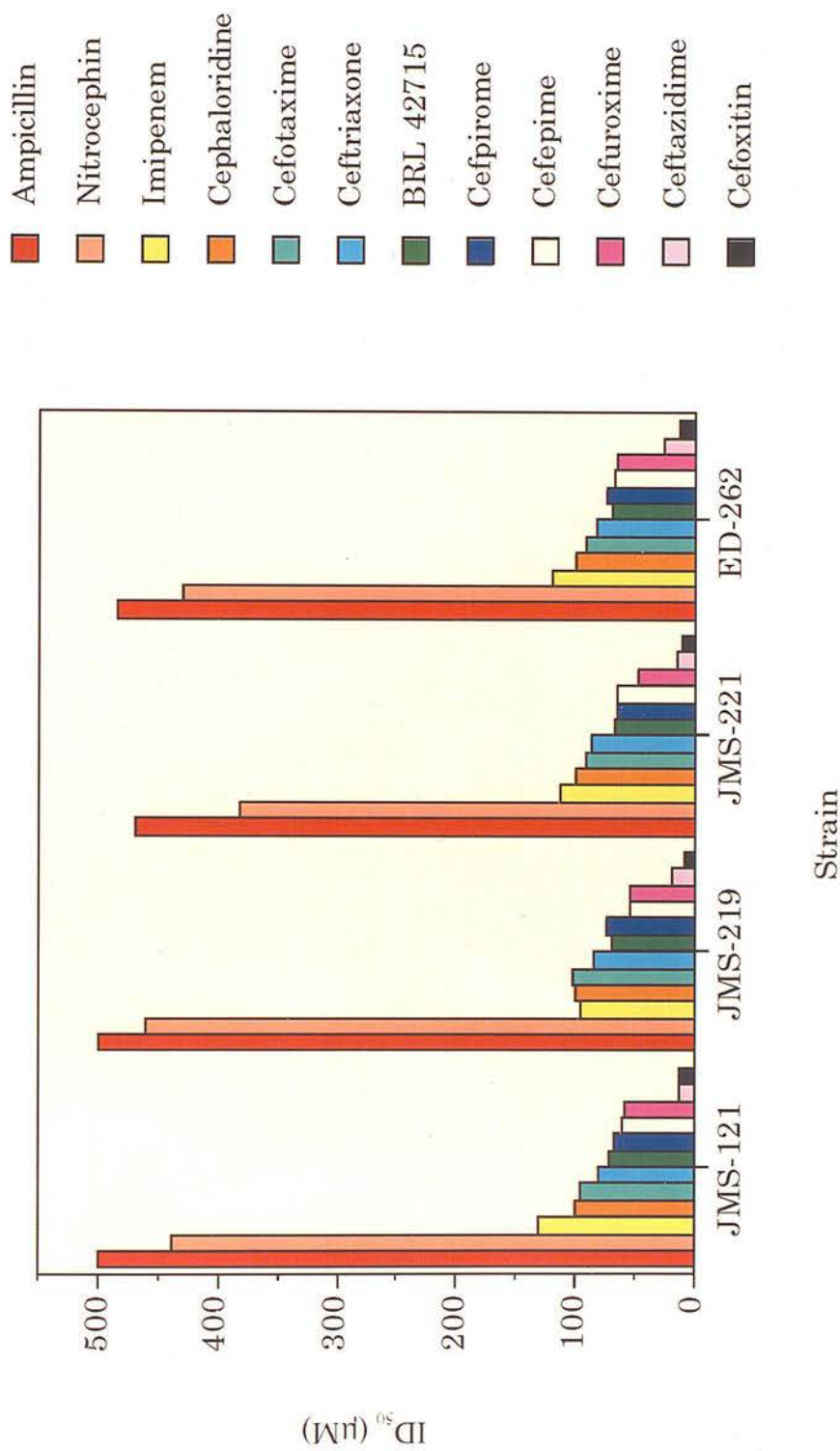
6.5. Biochemical Analysis of *Bact. fragilis* β -lactamases.

6.5.1. Substrate Profiles.

The substrate profiles of these enzymes for 12 different antimicrobials were then determined. Each of these compounds are known substrates for all metallo- β -lactamase known to date with the single exception of the CphA enzyme from *A. hydrophila*. The relative rates of hydrolysis of these β -lactams can be seen in Figure 6.11 overleaf.

All rates of substrate hydrolysis are plotted relative to cephaloridine, which was taken as 100%.

Figure 6.11: Substrate Profiles of Imipenem Hydrolysing *Bact. fragilis* Isolates



The substrate profiles of the β -lactamases from JMS-121, JMS-219 and JMS-221 were very similar to that of *Bact. fragilis* ED-262, a known CfiA producer. This suggested that the enzymes may all be CfiA-type metallo- β -lactamases. JMS-219 exhibited a slightly lower rate of hydrolysis of imipenem compared with the other enzymes, but this difference in rate (which is shown relative to cephaloridine) was not sufficient to suggest that this enzyme is unrelated to the others. It is possible that this difference may have been caused by a point mutation in the *CfiA* gene since several mutations have been reported between the enzymes sequenced to date, although these seem to have little effect on hydrolytic activity or substrate specificity. A list of the strains for which the CfiA type metallo- β -lactamase sequences have been reported, is shown in Table 1.4.

All these β -lactamases were able to hydrolyse the carbapenem meropenem as well as imipenem, although this data could not be compared directly to the other substrates, and hence are not shown in Figure 6.11.

6.5.2. Inhibitor Profiles.

Table 6.5 on the next page clearly shows that none of the clinically available β -lactamase inhibitors had any significant effect on these β -lactamases. These mechanism based inactivators have been designed and utilised to fight serine active site β -lactamases, and as such, show little or no effect on Zn^{2+} dependent metallo- β -lactamases.

Table 6.5: Inhibitor Profiles of Imipenem Hydrolysing β -lactamases from *Bact. fragilis* Isolates Against Clinical and Experimental Inhibitors.

Strain	Reporter Substrate	ID ₅₀ Values for Various β -lactamase Inhibitors (μ M)				
		Clavulanate	Aztreonam	Sulbactam	Tazobactam	BRL 42715
JMS-121	Nitrocephin	>1000	>1000	>1000	>1000	40
	Imipenem	>1000	>1000	>1000	>1000	3
JMS-219	Nitrocephin	>1000	>1000	>1000	>1000	30
	Imipenem	>1000	>1000	>1000	>1000	8
JMS-221	Nitrocephin	>1000	>1000	>1000	>1000	35
	Imipenem	>1000	>1000	>1000	>1000	4
ED-262 (CfIA-type)	Nitrocephin	>1000	>1000	>1000	>1000	45
	Imipenem	>1000	>1000	>1000	>1000	10
Bc II	Nitrocephin	>1000	>1000	>1000	>1000	30
NmcA	Nitrocephin	1	23	9	2	0.02

Bc II denotes the *Bacillus cereus* II metallo- β -lactamase.

NmcA denotes the serine active site carbapenemase from *Enterobacter cloacae* strain NOR-1.

The experimental penem BRL 42715 was the only compound with the ability to inhibit any of these β -lactamases at a physiologically attainable concentration. However, it should be noted that this compound has been shown to be a substrate for metallo- β -lactamases [28]. This is confirmed by the data shown in Figure 6.11. It is thus possible that this compound may be acting as a competitive substrate for these metallo- β -lactamases, and this manifests itself as an inhibition of hydrolytic activity.

Metal ion chelators, which sequester the divalent cation from the metallo- β -lactamase have been shown to be effective inhibitors of these enzymes *in vitro* but their indiscriminate ion chelation leads to toxicity, and hence precludes their use outside diagnostic experiments in the laboratory. Dipicolinic acid, *o*-phenanthroline and EDTA are all examples of such chelators, each of which show similar ID_{50} values for all the *Bact. fragilis* β -lactamases tested.

Table 6.6. Effects of Chelators on *Bact fragilis* β -lactamases.

Strain	ID_{50} Values for Various Chelators (μM)		
	Dipicolinic Acid	<i>o</i> -Phenanthroline	EDTA
JMS-121	30	30	1000
JMS-219	40	40	1000
JMS-221	60	50	1000
ED-262	40	30	1000
Bc II	70	110	990
NmcA	1000	>1000	>1000

Bc II denotes the *Bacillus cereus* II metallo- β -lactamase.

NmcA denotes the serine active site carbapenemase from *Enterobacter cloacae* strain NOR-1.

Table 6.7. Ability of Zinc to Restore β -lactamase Activity.

Strain	% Activity after 10mM EDTA o/n†	% Activity after 1mM Zinc (10mins)‡
JMS-121	0.8	22
JMS-219	0.2	20
JMS-221	0.3	21
ED-262	0.6	26
BcII	ND	78
TEM-1	98	97

† Activity after overnight dialysis against a 100-fold excess of 25mM PIPES pH 7.0 with 10mM EDTA at 4°C.

‡ Activity after a 10 minute preincubation of the EDTA dialysed sample in a 100-fold excess of 25mM PIPES pH 7.0 with 1mM zinc sulphate at 37°C.

BcII - *B. cereus* II metallo- β -lactamase.

Since these enzymes exhibited substrate and inhibitor profiles consistent with metallo- β -lactamases, more specifically, the CfiA type metallo- β -lactamase, and metallo- β -lactamases have been compared with other metal ion proteases, the ID₅₀ of captopril, an angiotensin converting enzyme inhibitor [217] was also determined. Angiotensin converting enzymes also employ a metal ion, in this case zinc as part of their active site.

Table 6.8: Effects of Other Compounds on *Bact fragilis* β -lactamases.

Strain	Substrate	ID ₅₀ Values for Other Compounds (μ M)		
		Captopril	pCMB	ZnSO ₄
JMS-121	Nitrocephin	>1000	11	No Effect
	Imipenem	ND	ND	1000
JMS-219	Nitrocephin	>1000	8	No Effect
	Imipenem	ND	ND	1000
JMS-221	Nitrocephin	>1000	12	No Effect
	Imipenem	ND	ND	1000
ED-262	Nitrocephin	>1000	16	No Effect
	Imipenem	ND	ND	1000
Bc II	Nitrocephin	200	ND	No Effect *
NmcA	Nitrocephin	>1000	ND	No Effect *

ND - Not Determined

Bc II denotes the *Bacillus cereus* II metallo- β -lactamase.

NmcA denotes the serine active site carbapenemase from *Enterobacter cloacae* strain NOR-1.

* No effect with nitrocephin as reporter substrate. ID₅₀ can be determined with imipenem as reporter substrate. See Chapter 5.

pCMB is an amino acid modifier which binds to cysteine residues in proteins. Since metallo- β -lactamases can employ histidine or cysteine residues for metal ion co-ordination, and CfiA employs three histidines and a cysteine residue for co-ordination, the inhibition by this compound further suggests that these enzymes are metallo- β -lactamases.

6.5.3. Kinetic Constants of *Bact. fragilis* β -lactamases.

A comparison of the enzyme kinetics of these β -lactamase was undertaken with imipenem as reporter substrate. These data are shown in Table 6.9 below.

Table 6.9: Activity Constants from *Bact. fragilis* β -lactamases.

Strain	K_m (μM) [‡]	V_{max} [‡] ($\mu mol/min/mg$)
JMS-121	350	0.31
JMS-219	380	0.41
JMS-221	385	0.38
ED-262	360	0.36

[‡] Imipenem as reporter substrate.

Together, the substrate, inhibitor, and kinetic profiles of these β -lactamases strongly suggest that the β -lactamase produced by the JMS- strains are CfiA related metallo- β -lactamases.

CHAPTER SEVEN

Carbapenemase Production by Clinical Isolates of the Genus *Flavobacterium*.

7.1. Prologue.

To date there has only been a single report describing the production of a metallo- β -lactamase from an isolate of the genus *Flavobacterium*. In this thesis it was postulated that other organisms from this genus may also produce metallo- β -lactamases. To investigate this, other clinical isolates of the genus *Flavobacterium* were examined for the production of a carbapenem hydrolysing β -lactamase, possibly a metallo- β -lactamase. Organisms of the genus *Flavobacterium* have been found both in natural ecosystems and in clinical specimens. A total of 10 *Flavobacterium* clinical isolates were received from the National Collection of Type Cultures (NCTC) in London, and examined for metallo- β -lactamase production. The NCTC catalogue numbers and sites of original isolation are listed in Table 7.1 below. The identities of all strains were confirmed by ATB32GN.

Table 7.1: *Flavobacterium* Isolates received from NCTC.

Species	NCTC Cat. No.	Site of Isolation
<i>F. breve</i>	11099	bronchial secretion
<i>F. gleum (indologenes)</i>	10795	sputum
<i>F. indologenes</i>	10796	trachea
<i>F. meningosepticum</i>	10016	spinal fluid
<i>F. multivorum</i>	11343	spleen
<i>F. odoratum</i>	11180	wound
<i>F. spiritivorum</i>	11388	blood
<i>F. thalpophilum</i>	11429	wound
<i>F. thalpophilum</i>	11430	abscess
<i>F. yabuuchiae</i>	12114	peritoneal fluid

7.2. Determination of Carbapenem Hydrolysis.

Initially, these strains were grown up in 10mls Nutrient Broth No. 2, with and without 0.5mg/l imipenem to act as inducer. Spectrophotometric analysis of these sonicated crude cell-free lysates with nitrocephin and imipenem as reporter substrates showed that the β -lactamase activities of these strains were expressed constitutively, since no significant increase in specific activity of any of these strains could be detected after induction. All but one of the preparations were able to cause significant imipenem hydrolysis. Additional experiments were conducted to further characterise this imipenem hydrolysis.

The rate of hydrolysis of imipenem by each crude preparation was determined. An aliquot of each sample was then assayed after a five minute pre-incubation with 1mM EDTA. This procedure was repeated with nitrocephin as reporter substrate. Table 7.2 below shows the rate of hydrolysis of imipenem by each sample, and the effect of 1mM EDTA on this hydrolysis.

Table 7.2: Effect of EDTA on Imipenem Hydrolysis of *Flavobacterium* Isolates.

Species	NCTC Cat. No.	MIC [§] (mg/l)	Rate of Imipenem Hydrolysis (μ M/min)	% Activity with 1mM EDTA
<i>F. indologenes</i>	10796	8	56.5	67
<i>F. meningosepticum</i>	10016	4	31.8	56
<i>F. breve</i>	11099	0.25	16.3	48
<i>F. gleum (indologenes)</i>	10795	4	10.2	16
<i>F. spiritivorum</i>	11388	4	7.6	36
<i>F. odoratum</i>	11180	1	6.7	68
<i>F. thalpophilum</i>	11429	1	4.7	100
<i>F. multivorum</i>	11343	2	2.9	63
<i>F. yabuuchiae</i>	12114	2	2.1	64
<i>F. thalpophilum</i>	11430	2	0.81	-

[§] Minimum inhibitory concentration of imipenem.

In all but two of the isolates imipenem hydrolysis was partially inhibited by the pre-incubation of the enzymes with 1mM EDTA suggesting that these isolates may produce a metallo- β -lactamase, in some cases in addition to a serine active enzyme. This EDTA inhibition was measured after a 5 minute pre-incubation with this chelator. When repeated after a 10 minute pre-incubation with EDTA prior to the assays, similar data were obtained, suggesting that all inhibition of enzyme activity had occurred within the first 5 minutes of pre-incubation.

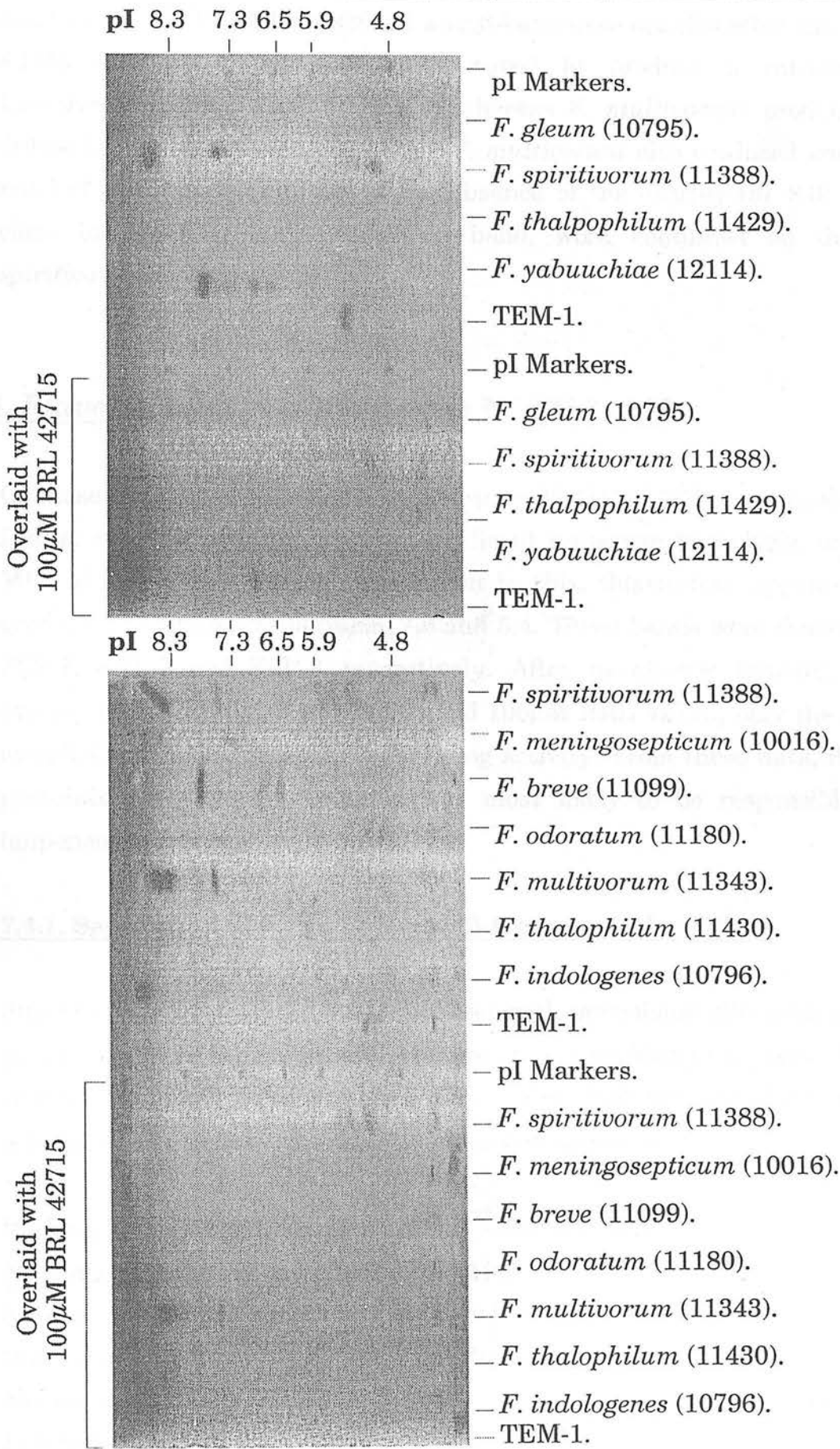
7.3. Separation of *Flavobacterium* β -lactamases by IEF.

The β -lactamases of these strains were separated by IEF, to determine whether this imipenem hydrolysis was mediated by the production of an enzyme similar to that found in the imipenem resistant *F. odoratum* strain previously reported. To indicate this, the enzymes were separated by IEF, and the gel was overlaid with 1mM ZnSO_4 for 5 minutes. Duplicate loadings were overlaid with 100 μM BRL 42715, since this is known to be a potent inhibitor of all serine active site β -lactamases. It was postulated that if present, a metallo- β -lactamase would retain β -lactamase activity in the presence of this compound, and hence still hydrolyse the nitrocephin present in the gel overlay. Possible metallo- β -lactamases could thus be identified separately from serine active site β -lactamases.

Iso-electric focusing of these extracts revealed between 1 and 3 bands when stained with nitrocephin. Two strains (*F. indologenes* and *F. gleum* (*indologenes*)) both appeared to produce one β -lactamase with $\text{pI} > 9.0$. The *F. spiritivorum* and *F. meningosepticum* strains both appeared to produce 3 bands by IEF at pI 5.4, 7.6 and 8.6.

Figure 7.1 overleaf shows the nitrocephin stained IEF gel of these isolates, with and without the 100 μM BRL42715 overlay.

Figure 7.1: IEF Showing *Flavobacterium* β -lactamases.



Of the strains examined, only *F. spiritivorum* NCTC 11388 and *F. multivorum* NCTC 11343 retained any β -lactamase activity after the BRL 42715 overlay. *F. spiritivorum* appeared to produce a nitrocephin hydrolysing doublet band at pI 5.4, whereas *F. multivorum* produced a diffuse band of high pI (pI~9.0). Since *F. multivorum* also produced another band of β -lactamase activity in the absence of the overlay (pI 8.0) very close to the BRL 42715 resistant band, work continued on the *F. spiritivorum* isolate.

7.4. *Flavobacterium spiritivorum* NCTC 11388.

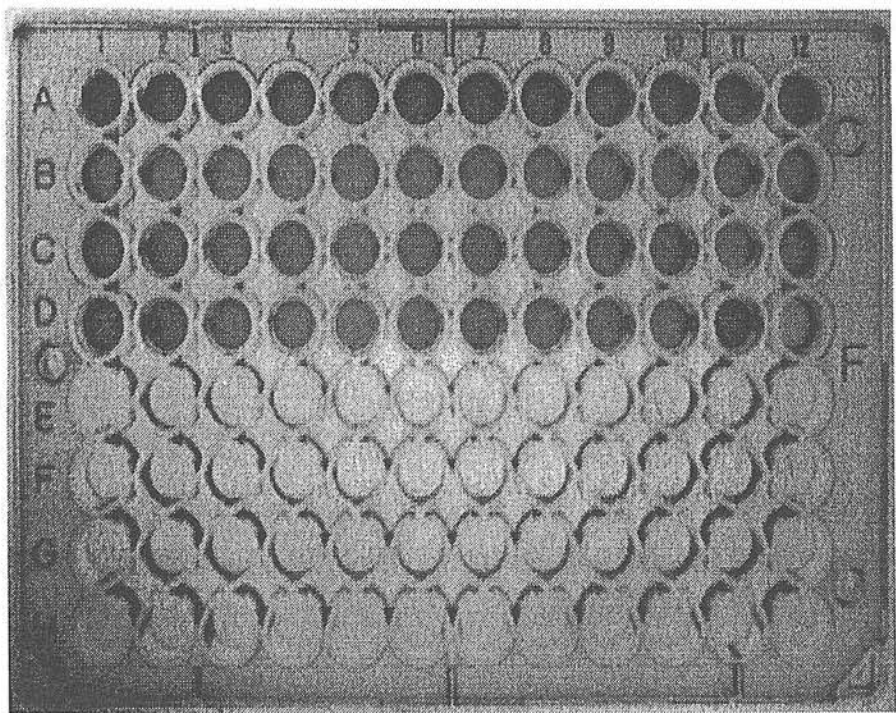
Of these isolates, one strain; *F. spiritivorum* NCTC 11388 was chosen for further study. This strain showed significant imipenem hydrolysis, and an MIC of imipenem of 4mg/l. In addition to this, this isolate appeared to produce 3 β -lactamases of pI 8.6, 7.6 and 5.4. These bands were designated FSP-1, FSP-2 and FSP-3 respectively. After iso-electric focusing, and overlay of the gel with 1mM ZnSO₄ and 100 μ M BRL 42715, only the band at pI 5.4 retained nitrocephin hydrolysing activity. From these data, it was postulated that the pI 5.4 band was most likely to be responsible for imipenem hydrolysis.

7.4.1. Separation of *F. spiritivorum* β -lactamases by FPLC.

Since this strain produced 3 β -lactamases with provisional allocation of the pI 5.4 enzyme as the metallo- β -lactamase, it was decided to separate these enzymes by FPLC. To this end, a separation methodology was developed to separate this enzyme from the other two β -lactamases.

Initially, a crude cell free extract of this strain was tested against the 46 ion exchange matrices as described in Materials and Methods. However, no hits were observed by this method. This may have been caused by the fact that this isolate appeared to produce 3 β -lactamases by nitrocephin stained IEF, and no single matrix bound all three enzymes simultaneously as shown in Figure 7.2 overleaf.

**Figure 7.2: Ion Exchange Matrix Screen of *F. spiritivorum*
NCTC 11388.**



Rows E to H were not loaded.

Since the ion exchange matrix screen did not yield any 'hits', several small scale matrices were set up in an attempt to resolve these enzymes.

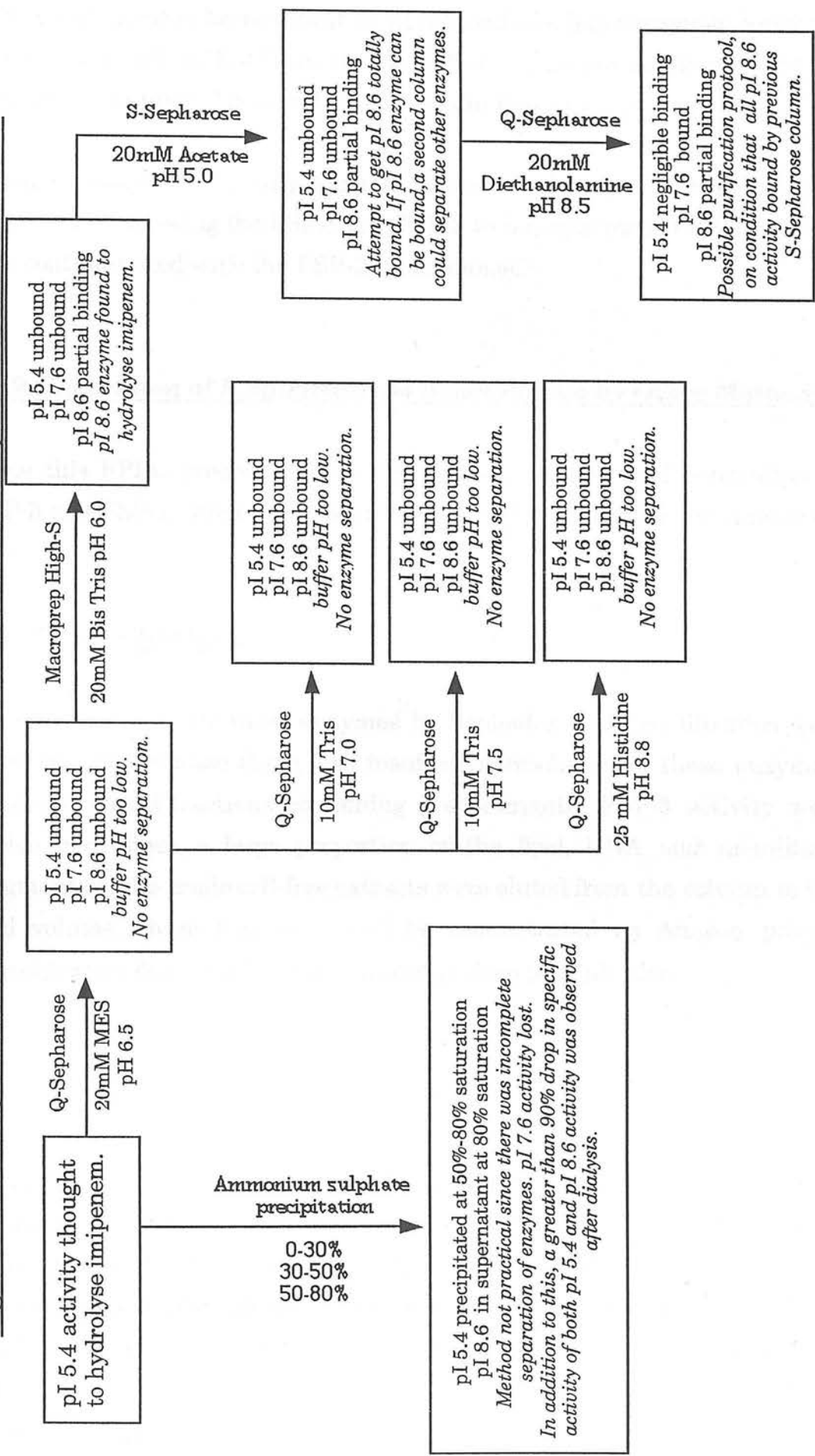
Figure 7.3 overleaf shows a summary of the purification methodology, and the steps involved in its development. Attempts to bind FSP-3 were unsuccessful, at any of the pH values tested. As a result of this, the anion matrix had to be substituted with a cation exchange matrix.

Each of these matrices were set up as a small scale column with a bed volume of 20mls and equilibrated at the appropriate pH with elution buffer. Fractions were eluted in a NaCl step gradient 0M, 0.25M, 0.5M 0.75M and 1M with respect to sodium chloride as described in Materials and Methods. Eluted fractions were assayed for nitrocephin hydrolysis, and sensitivity to inhibition by clavulanic acid and EDTA in an attempt to determine the class of β -lactamase eluted. Active fractions were also separated by IEF.

A MacroPrep High S column was set up in Bis Tris pH 6.0 in an attempt to bind FSP-1 and FSP-2. These enzymes were separated first, since this column would bind proteins of high pI, and bacteria produce fewer proteins of high pI, than low pI. The pI 8.6 β -lactamase partially bound to this column, when assayed, was found to hydrolyse imipenem.

This purification protocol was altered, such that the crude extract would be passed through a cation exchange column to bind the high pI enzymes, followed by an anion exchange column to bind the other enzymes. This purification system did resolve FSP-2 which bound to the Q-Sepharose column, and the FSP-3 which failed to bind to either matrix. However, insufficient β -lactamase activity was purified to complete the biochemical analysis of these β -lactamases.

Figure 7.3: Development of Purification Protocol for *Flavobacterium spiritivorum* β -lactamases.



FSP-2 was found to be resistant to EDTA dialysis, but extremely sensitive to inhibition with BRL 42715. However, FSP-3 was not inhibited by either of these compounds. These data are shown in Figure 7.5 on page 142.

When repeated, FSP-2 only partially bound to the Q-Sepharose column, which whilst allowing the FSP-1 and FSP-3 to be separated, FSP-3 activity was contaminated with the FSP-2 β -lactamase.

7.4.2. Separation of *F. spiritivorum* β -lactamases by Other Methods.

Since this FPLC procedure led to significant, but not total separation of FSP-3 and FSP-2, other biochemical methods of separation were examined.

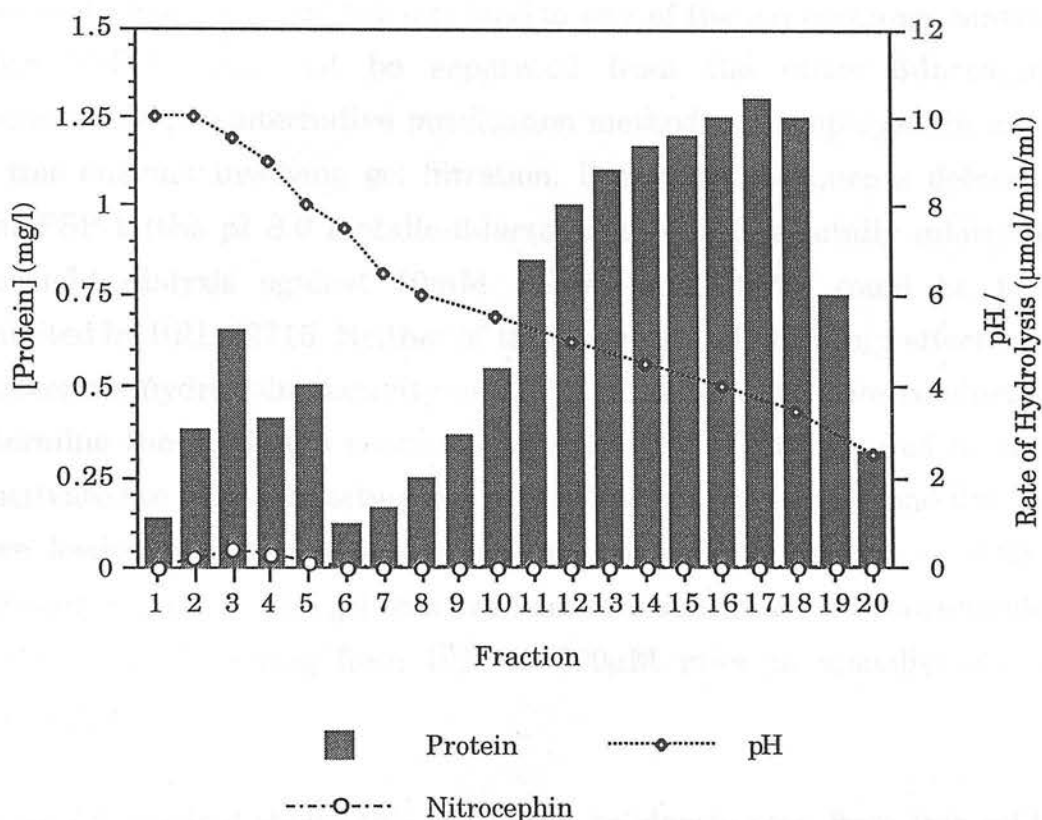
7.4.2.1. Gel Filtration.

Attempts to separate these enzymes by Sephadex G150 gel filtration were unsuccessful, because there was insufficient resolution of these enzymes. However, eluted fractions containing predominantly FSP-3 activity were pooled and, since a large proportion of the lipid, DNA and membrane fragments of the crude cell-free extracts were eluted from the column in the void volume, these fractions could be concentrated on Amicon protein concentrators from 25ml to 2ml without protein precipitation.

7.4.2.2. Free-Flow and Preparative IEF.

Attempts to separate these β -lactamases by free-flow IEF were unsuccessful, since only insubstantial β -lactamase activity could be detected in any fractions after separation. Figure 7.4 shows the activity of the 20 fractions eluted after MinipH_{or} free-flow IEF. After separation, a very low level of nitrocephin hydrolysis was detected in the fractions containing proteins of high pI. No imipenem hydrolysis was detected in any of the eluted fractions.

Figure 7.4: β -lactamase Activity of Fractions Eluted from MinipHor Free-Flow IEF.



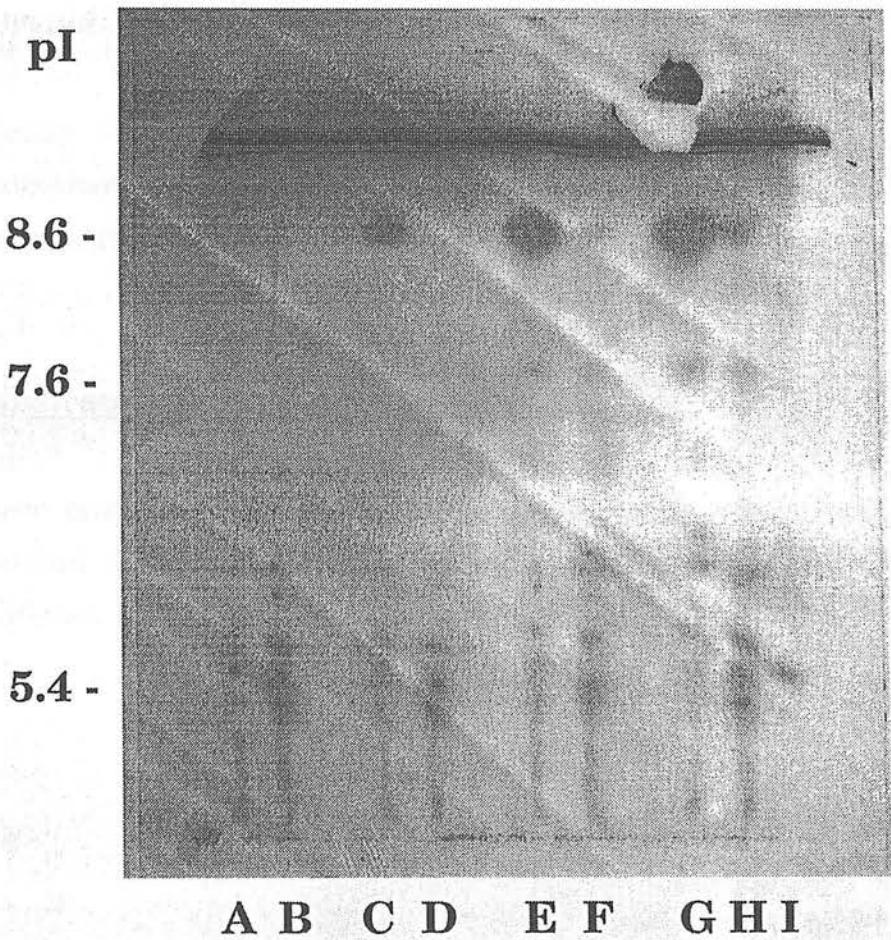
One millilitre of the concentrated sample from Section 7.4.2.1 was loaded onto lanes 2-11 of a 12 lane analytical poly-acrylamide IEF gel pH 3.5-9.5. Lanes 1 and 12 contained TEM-1 (pI 5.4) to act as a pI marker and control. Once run, only lanes 1, 2 and 12 were visualised with 500mg/l nitrocephin. The regions of the gel in lanes 3-11 which corresponded to FSP-3 (pI 5.4) were excised, and eluted from the gel slice by electroelysis. However, no β -lactamase activity could be detected from the sample eluted from the gel. Staining of the remainder of the IEF gel and gel slice failed to locate FSP-3, suggesting that it had been lost during electroelution.

7.4.2.3. Combination of Gel Filtration and Selective Inactivation.

FSP-1 and FSP-2 could be separated by FPLC. However, FSP-3 could not be eluted separately, and failed to bind to any of the ion exchange matrices. Since FSP-3 could not be separated from the other β -lactamases biochemically, an alternative purification method was employed for assays on this enzyme involving gel filtration. Previous experiments determined that FSP-1 (the pI 8.6 metallo- β -lactamase) could be totally inhibited by overnight dialysis against 10mM EDTA, and FSP-2 could be totally inhibited by BRL 42715. Neither of these compounds had any effect on the nitrocephin hydrolysing activity of FSP-3. Experiments were conducted to determine the minimum concentration of BRL 42715 required to totally inactivate the FSP-2 β -lactamase. An IEF gel was prepared, and five lanes were loaded with the concentrated fractions from Sephadex G150 gel filtration. Once run, the gel was overlaid for 5 minutes with concentrations of BRL 42715 varying from 1 μ M to 100 μ M prior to visualisation with nitrocephin.

Figure 7.5 overleaf shows this gel. It can be clearly seen from this gel that 10 μ M BRL 42715 effects total enzyme inhibition. This concentration was sufficiently low that this inhibitor could be present in the assay solution without adversely affecting the measurement of substrate hydrolysis.

**Figure 7.5: IEF Gel Showing Minimum Concentration of BRL 42715
Required to Inhibit FSP-2 from *F. spiritivorum*.**



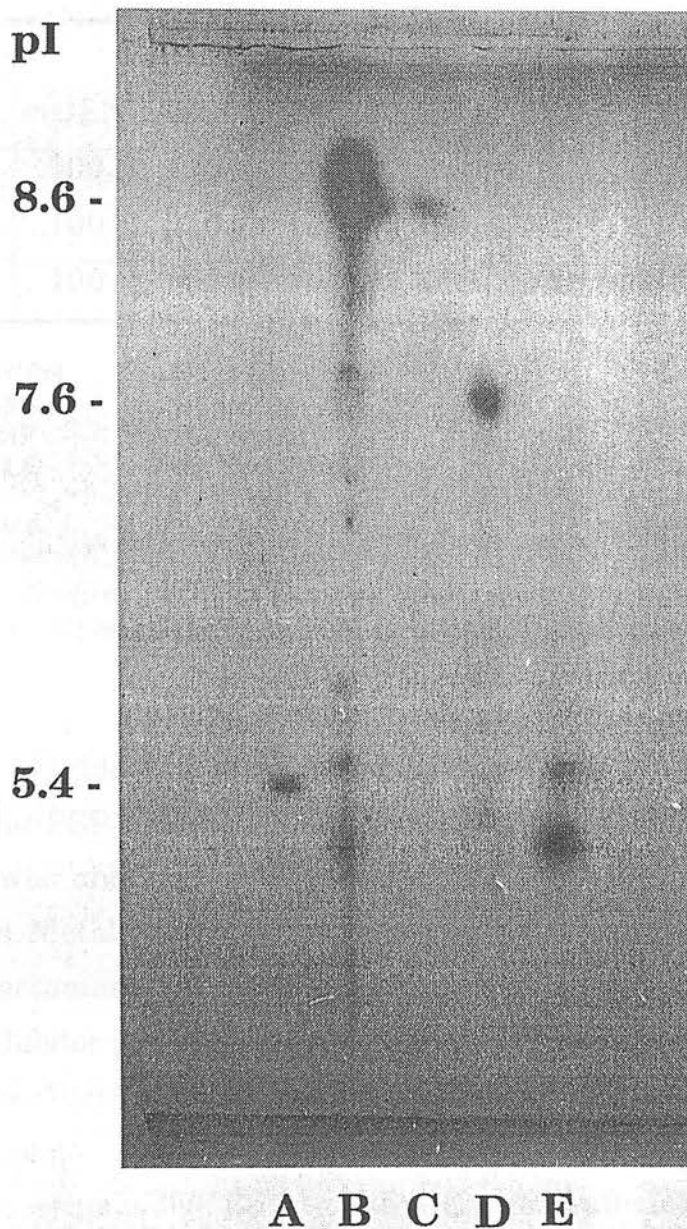
- | | |
|--------|--|
| Lane A | G150 eluent of <i>F. spiritivorum</i> overlaid with 100 μ M BRL 42715. |
| Lane B | As lane A pre-incubated with 10mM EDTA overnight at 4 $^{\circ}$ C. |
| Lane C | G150 eluent of <i>F. spiritivorum</i> overlaid with 10 μ M BRL 42715. |
| Lane D | As lane C pre-incubated with 10mM EDTA overnight at 4 $^{\circ}$ C. |
| Lane E | G150 eluent of <i>F. spiritivorum</i> overlaid with 1 μ M BRL 42715. |
| Lane F | As lane E pre-incubated with 10mM EDTA overnight at 4 $^{\circ}$ C. |
| Lane G | G150 eluent of <i>F. spiritivorum</i> (no BRL 42715 overlay). |
| Lane H | As lane G pre-incubated with 10mM EDTA overnight at 4 $^{\circ}$ C. |
| Lane I | TEM-1. |

Thus, dialysis of the sample against 10mM EDTA followed by the addition of 10 μ M BRL 42715 to the assay mixture totally inhibited both the FSP-1 and FSP-2 enzymes respectively. This allowed the analysis of FSP-3 without contamination from the other enzymes even though the sample contained the other (inactivated) β -lactamases. Figure 7.6 overleaf shows an iso-electric focusing gel of a crude preparation of *F. spiritivorum* NCTC 11388 together with the enzymes which had been separated by the procedures described previously.

7.4.3. Substrate and Inhibitor Profiles of FSP-1, FSP-2 and FSP-3.

Once these enzymes had been resolved biochemically, each enzyme was examined and characterised separately. Table 7.3 describes the substrate profiles of each of these enzymes.

Figure 7.6: Separated β -lactamases from *F. spiritivorum* NCTC 11388.



- | | |
|--------|--|
| Lane A | TEM-1 (pI 5.4). |
| Lane B | Crude <i>F. spiritivorum</i> NCTC 11388. |
| Lane C | FSP-1 (pI 8.6). |
| Lane D | FSP-2 (7.6). |
| Lane E | FSP-3 (5.4) with 10 μ M BRL 42715 overlay. |

Table 7.3: Substrate Profiles of β -lactamases Produced by *F. spiritivorum* (NCTC 11388).

Enzyme	Relative Rate of Hydrolysis (Nitrocephin taken as 100%)						
	NCFN	AMP	IMP	CLD	CFX	CTX	CZD
FSP-1	100	425	18	6	6	ND	ND
FSP-2	100	270	ND	ND	ND	ND	ND
FSP-3	100	ND	ND	ND	ND	ND	ND

NCFN - nitrocephin
 AMP - ampicillin
 IMP - imipenem
 CLD - cephaloridine

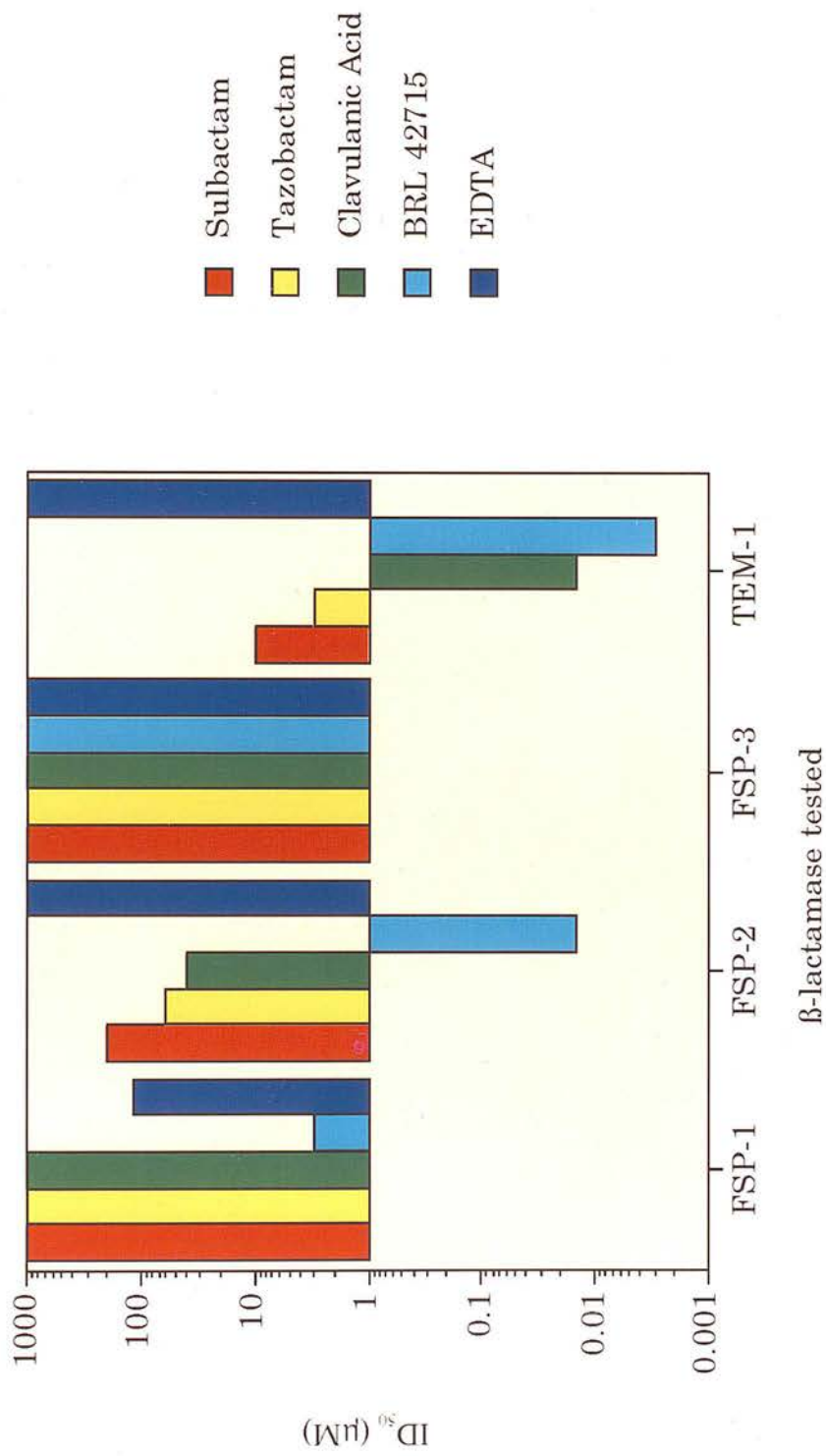
CFX - cefuroxime
 CTX - cefotaxime
 CZD - ceftazidime

ND - None Detected.

The substrate profiles in the above table were expressed relative to nitrocephin, since all enzymes were able to hydrolyse this substrate.

The substrate [Table 7.3 above] and inhibitor profiles [see Figure 7.7 overleaf] for the FSP-1 enzyme were indicative of a metallo- β -lactamase. This enzyme was able to hydrolyse imipenem, ampicillin, and a range of cephalosporins. Metallo- β -lactamases are generally regarded as resistant to all clinical β -lactamase inhibitors including clavulanic acid. FSP-1 shows this type of inhibitor profile, and only BRL 42715 was able to inhibit this enzyme at a measurable concentration. The ID_{50} of this compound is in the micro-molar range, which is consistent with that of other metallo- β -lactamases. Serine active β -lactamases are generally inhibited by nano-molar concentrations of this compound as stated in Chapter 1, and Figure 7.7. This is borne out by the ID_{50} values for the TEM-1 β -lactamase which have been included in this table, both as a control and for comparative purposes.

Figure 7.7 : Inhibitor Profiles of β -lactamases from *F. spiritivorum* (NCTC 11388)



The inhibition data for the FSP-2 enzyme suggested that this was a serine active site β -lactamase and by virtue of its pI and its substrate / inhibitor profiles [Table 7.3 and Figure 7.7] may be SHV or OXA derived. Once purified by FPLC, the specific activity of this enzyme was very low, and no hydrolysis of any substrates other than nitrocephin and ampicillin could be detected spectrophotometrically.

FSP-3 was very unusual. This enzyme did not bind to any of the ion exchange matrices, at any of the pH values tested. This is uncommon, and more work would be needed to improve the separation protocol to yield a greater specific activity to facilitate the completion of the biochemical analysis of this protein. This protein was found to be able to hydrolyse nitrocephin at a low rate, but no hydrolysis of any other substrate could be detected, even after several hours incubation. In addition to this, none of the β -lactam antibiotics or inhibitors tested including BRL 42715 were able to inhibit the nitrocephin hydrolysis by this enzyme. The possible reasons for this are discussed in a later section.

7.4.4. Molecular Mass Estimations.

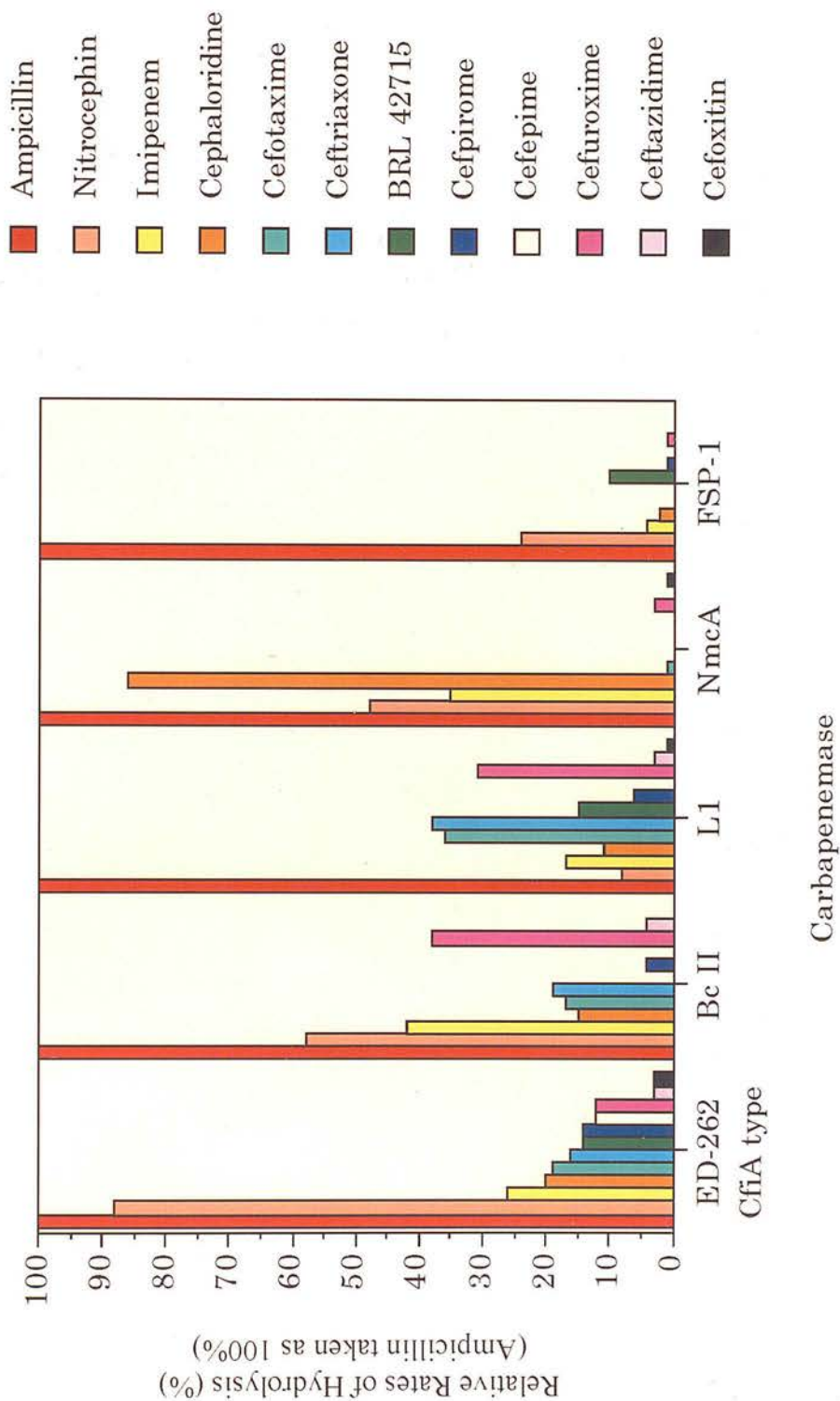
Sephadex G150 gel filtration of a crude cell-free extract of *F. spiritivorum* NCTC 11388 suggested that the molecular weights of FSP-1, FSP-2 and FSP-3 were 33kDa, 27kDa and 21kDa respectively, but did not lead to sufficient enzyme separation to allow further analysis.

7.4.5. Comparison of FSP-1 with Known Carbapenemases.

Since FSP-1 showed a substrate and inhibitor profile which was indicative of a metallo- β -lactamase, the relative rates of hydrolysis of various substrates by this enzyme were compared with several other known metallo- β -lactamases including a *Bact. fragilis* CfiA-type enzyme, *Sten. maltophilia* L1, and *B. cereus* II. The serine active site carbapenemase NmcA from *Enterobacter cloacae* was also included in this comparison. This

comparison is shown in Figure 7.8. These carbapenemases each showed broad substrate profiles, hydrolysing penicillins, cephalosporins as well as carbapenems. Despite this, each of these enzymes show a very distinct hydrolytic pattern for the various substrates. By virtue of its pI, inhibitor profile, and the observation that its substrate profile is distinct from other carbapenemases, I propose that this enzyme is a novel carbapenemase, and probably a metallo- β -lactamase.

Figure 7.8 : Substrate Profiles of Several Carbenemases Including FSP-1 from *F. spiritivorum*



7.4.6. Epilogue.

The separation of these three enzymes demonstrated the objectivity required in the analysis of strains producing several β -lactamases. Initial overlays of the IEF gels with BRL 42715 suggested that the pI 5.4 enzyme was the metallo- β -lactamase.

Small clues starting with the imipenem hydrolysing activity of the pI 8.6 enzyme [Section 7.4.1] suggested that the initial observations and subsequent hypotheses might be inaccurate. Full separation and analysis of the β -lactamases revealed the imipenem hydrolysing metallo- β -lactamase with a pI 8.6.

CHAPTER EIGHT

Discussion.

8.1. Carbapenemases and Divalent Cations.

8.1.1. Purification Methodology.

Many different techniques have been tried in the process of metallo- β -lactamase purification to separate these enzymes from other β -lactamases or cell debris. These methods include gel filtration [139], free-flow IEF [128], ion exchange chromatography [146, 151], FPLC [140], affinity chromatography [103], selective precipitation [164] or combinations of these [152] [this thesis]. Whilst many of these techniques can be applied to the partial purification of a single protein, there are few reports comparing these techniques for a given system. There is no evidence to suggest that any particular system has been optimised. An example of this can be seen in [134] where Tris buffer was prepared and the pH determined at room temperature, yet this buffer was used at temperatures between 0°C and 30°C. The change in pKa per degree Celsius for Tris buffer is very high ($\Delta pK_a/C^\circ = -0.031$). For this reason Tris buffer pH 7.0 made at 25°C and chilled to 4°C will drop by 0.65 pH units to 6.35.

Arstila and co-workers examined five different methods for preparing crude cell-free lysates [218], but this report did not extend to methods of β -lactamase purification. The screening of 46 different ion exchange matrices undertaken in this thesis does go some way toward this optimisation. This screen encompassed matrices from all major groups, including anion, cation, affinity and agarose matrices. In the case of the *Bact. fragilis* metallo- β -lactamase purification screens, the MacroPrep High-Q, a high capacity anion exchange matrix showed greatest

β -lactamase binding. The ω -amino hexyl sepharose matrix, also showed good β -lactamase binding. This latter matrix had been used by Felici and co-workers during their purification of the *A. hydrophila* A2 metallo- β -lactamase [103]. However this system has two potential disadvantages:

1. Such a matrix relies on the fact that the metallo- β -lactamase will adsorb onto the zinc which was bound to the matrix support. This binding will depend on the K_d of zinc in the active site of such an enzyme. If the metallo- β -lactamase binds this zinc poorly, then total adsorption to the matrix is unlikely.
2. Once adsorbed onto the column, elution of the metallo- β -lactamase with an increasing sodium chloride gradient will leave the metallo- β -lactamase temporarily without a zinc ion in the active site. It is possible that once removed, the protein could undergo a conformational change or become denatured in such a way that could not be totally renatured by the later addition of zinc (if eluted with NaCl), or presence of zinc (if eluted with NaCl and zinc). It is possible that once the divalent cation has been removed from the active site, the ensuing conformational change could prevent another ion from associating with the enzyme. The resulting 'zinc-less' metallo- β -lactamase would show negligible β -lactamase activity. This dissociation constant will vary between metallo- β -lactamases, and so certain enzymes, where the recoverability of hydrolytic activity after EDTA dialysis is low (e.g. *Bact. fragilis* CfiA-type enzymes), would be more prone to such inactivation than those with high recovery after this treatment (e.g. *B. cereus* II) [see Table 6.7].

8.1.2. Assay Conditions.

For the study of serine active site β -lactamases, many workers have prepared these enzymes and conducted spectrophotometric assays in phosphate buffer (usually 25mM or 50mM) at pH 7.0, a well known and established general purpose buffer. This buffer is frequently used for assays involving β -lactamases as well as other enzyme systems. The pH of this buffer is defined by the ratio of PO_4^{2-} and HPO_4^- ions [219]. Once defined, the pH is maintained by an equilibrium between these two ions.

However, when metallo- β -lactamases were discovered, rather than re-evaluate the best buffer for these enzymes, the already established dogma of phosphate buffer was simply applied to these enzymes, and many workers have conducted their studies in phosphate buffer, but supplemented this buffer with 1mM zinc, usually in the form of ZnSO_4 [134] or ZnCl_2 [216] to accommodate the divalent metal ion requirements of metallo- β -lactamases. This assumption is invalid.

Zinc, in the form of Zn^{2+} is a small ion with an overall 2+ charge, making it a closed-shell 'hard' metal ion. As such, this ion acts as a strong Lewis acid, and will co-ordinate and bind to 'hard' donors, such as negatively charged forms of oxygen. Hard donors can, for this reason be regarded as chelators. Hard donors will include HPO_4^- and PO_4^{2-} ions. In a solution of phosphate buffer to which zinc sulphate has been added, the Lewis acid will become co-ordinated with hard donor ions. Zn^{2+} and phosphate ions both form tetrahedral 'diamond-shaped' complexes, and will co-ordinate in this fashion. However, all zinc phosphate compounds including zinc orthophosphate namely $\text{Zn}_3(\text{PO}_4)_2$ and zinc hydrogen orthophosphate namely $\text{Zn}(\text{H}_2\text{PO}_4)_2$ are insoluble ($S \sim 10^{-8} \text{ M}$), even in their octahydrate or tetrahydrate forms [220]. These complexes will associate together and grow into a polymeric crystal structure as a direct result of their low solubility. This will result not only in an overall drop in the Zn^{2+} concentration in the buffer but the growth of these crystals will manifest itself as a fine white flaky precipitate. This will have several implications on preparation steps and assays which include phosphate buffer with a zinc sulphate supplement:

1. Such a precipitate will cause significant light scattering in any spectrophotometric cell, and will lead to inaccurate and inconsistent readings of Δ -absorbance. This will affect the calculation of rates of substrate hydrolysis.
2. Since phosphate ions can be regarded as chelators, the effective concentration of zinc in the buffer will fall to the nanomolar range. Such chelation will mislead workers who have employed a specific zinc concentration in their buffer to gain maximal metallo- β -lactamase activity.
3. The loss of phosphate ions from the buffer will result in a small change in buffer pH as the ratio of HPO_4^- and PO_4^{2-} ions (which defines the pH) changes and hence the ionic strength of the buffer will also change. In addition to this, the concentration of the buffer will drop from its original concentration (e.g. 25mM or 50mM) with respect to phosphate ions. Since zinc and phosphate ions will precipitate, an excess of negatively charged anions will form; for example SO_4^{2-} and 2Cl^- (if the zinc was added to the buffer as zinc sulphate or zinc chloride respectively). The presence of these ions will affect not only the ionic strength of the buffer, but also the pH. The degree to which the buffer will be affected is unknown, but in the vast majority of cases the buffer concentration (usually 25mM or 50mM) will be significantly greater than the concentration of the zinc supplement. Nevertheless, these effects cannot be discounted.
4. The zinc ions co-ordinated as part of the active site of a metallo- β -lactamase are in dynamic equilibrium with the zinc ions in the surrounding buffer. Hence, the chelating effect of the phosphate ions will not only extend to the zinc present as a buffer supplement, but will also have a leaching effect on the zinc co-ordinated to the active site possibly causing enzyme inactivation after prolonged exposure.

Metallo- β -lactamases from different species vary considerably in their ability to bind zinc ions. The activity of *B. cereus* II can be almost totally inhibited by dialysis of the enzyme in a buffer without a zinc supplement or external chelating agent, for example EDTA. This inhibition can be partially restored by the addition of excess zinc sulphate at a later time, although the degree to which activity could be restored varied from enzyme to enzyme. In the case of *B. cereus* II [164] and *Sten. maltophilia* L1 [151], greater than 90% of enzyme activity can be restored by the introduction of a zinc supplement into the assay buffer [see Table 6.7]. Conversely, metallo- β -lactamases from other species, e.g. CfiA-type enzymes from *Bact. fragilis* were unaffected by dialysis unless an external chelating agent was present to sequester the zinc ion from the active site. In these cases, the restoration of activity after the re-introduction of zinc was found to be between 22% and 25% [Table 6.7] although figures from 45% [216] to 112% [138] have been reported. This observation could be explained by a dissociation constant k_d , describing the binding strength of the metallo- β -lactamase for zinc. If this were true, then *B. cereus* II would have a low k_d for zinc, suggesting a dynamic equilibrium between the ion in the active site and those in the surrounding buffer. The k_d for the *Bact. fragilis* metallo- β -lactamase would be significantly greater, since dialysis in buffers without zinc or chelators had a negligible effect on enzyme activity.

Whilst the 'hydrolysis' of β -lactam antibiotics by zinc ions in several buffer systems [118, 221-225] and bacteriological media [226] is widely known, little work has been conducted on the effects of zinc on the catalytic activity of β -lactamases, apart from the observation that the sequestration of this ion from the metallo- β -lactamase by EDTA and other chelators results in partially reversible enzyme inactivation.

Since it was first shown in 1966 that *B. cereus* II required zinc for activity [164], several other metallo- β -lactamases have been characterised [see Table 1.4]. In every case where the co-ordinated divalent cation has been identified, Zn^{2+} has been found to be the co-ordinated ion. Several workers have substituted this ion in different metallo- β -lactamases with other divalent cations including other transition metal ions and Group II ions.

These ions include Co^{2+} , Mg^{2+} , Mn^{2+} and Hg^{2+} [116, 151, 227]. Of these, cobalt has been shown to maintain greatest metallo- β -lactamase activity (after zinc) [122].

Davies and Abraham [122] also found that the addition of zinc increased the hydrolytic activity of *B. cereus* II against cephalosporin C, the only substrate tested, but did not observe enzyme inhibition. The only work which suggested any investigation into the determination of the optimum zinc concentration present in the buffer for metallo- β -lactamase activity was made in 1993 by Felici and co-workers who conducted assays on *B. cereus* II in buffer supplemented with 0.3mM ZnCl_2 , whereas all other metallo- β -lactamases were assayed in buffer with 0.1mM ZnCl_2 [103]. However, no explanations were given for the choice of these zinc concentrations.

One report by Hawkey and co-workers [214] investigated the effects of various divalent cations including zinc naturally present in the bacteriological media on the susceptibility to imipenem of several *Sten. maltophilia* isolates. This study concentrated on the microbiological effects of these ions, and described a correlation ($P=0.003$) between increased imipenem MICs and increased zinc ion concentration in the bacteriological media. This was attributed to the increased zinc concentration causing increased activity of L1-type metallo- β -lactamases in the isolates tested. This group also found that glassware which included metal caps and rubber inserts led to an increase in zinc concentration, especially where the rubber was in poor condition. For this reason, all work on the effects of cations on carbapenemase activity was conducted in disposable glassware with plastic caps wherever possible. Where there was no alternative, re-usable glassware was washed in 0.1M EDTA to remove any free ions which may be present after washing. A 0.4M NaOH rinse was also used to remove any detergent from the glassware which may sequester such ions. The glassware was then rinsed in MilliQ water until the pH returned to neutral to ensure removal of all compounds involved in these wash steps. No other work in the current literature describes these considerations, or has reported to have taken such extensive precautions.

All β -lactamase assays in this thesis, whether on suspected metallo- β -lactamases or serine active enzymes were conducted in 25mM PIPES buffer pH 7.0. Zinc supplements were added where applicable. This buffer was chosen for a variety of reasons:

1. PIPES is zwitterionic, and hence the ionic strength will not change with varying pH. The pK_a of PIPES is 6.8 at 20°C.
2. PIPES buffer has been shown to be stable in the presence of zinc ions, both moieties remaining in solution. PIPES shows low zinc binding when compared with other buffers including Tris, Ada and Bis Tris [224]. However, since PIPES is zwitterionic, there will be a small amount of zinc co-ordination, but since this complex remains in solution and forms a dynamic equilibrium, the effect on free zinc ion concentration will be minimal. 25mM PIPES buffer with a 10mM zinc sulphate supplement is stable for short periods [this thesis]. PIPES buffer with lower zinc sulphate concentrations was stable for prolonged periods.

For these reasons, PIPES buffer is ideal for the study of β -lactamases including metallo- β -lactamases, without the problems associated with phosphate buffer, outlined above.

An investigation into the effects of these zinc supplements on carbapenemase activity was undertaken in this thesis. By preparing a single culture of a metallo- β -lactamase producer, it was possible to compare directly the effects of this ion during preparation and activity determinations. It appeared that there was little difference in preparing metallo- β -lactamases in the absence of zinc sulphate, or in the presence of 1 μ M zinc sulphate (BFR_0 and BFR_1) as shown in Figures 5.2 and 5.3. Both preparations demonstrated similar specific activities for all substrates tested. Both preparations showed an *activation* as the zinc supplement in the assay buffer increased. With cephaloridine and nitrocephin as reporter substrates, the activation appeared to be linear as the log of zinc concentration increased to 1mM. However, with imipenem as substrate,

this activation was more marked, and peaked at 100 μ M ZnSO₄. Intriguingly carbapenemase activity fell back as the zinc concentration increased towards 1mM.

The interesting observation made during these experiments, was the greater specific activity of BFR₁₀₀₀ compared with BFR₀ and BFR₁ for all substrates tested. This increased specific activity in BFR₁₀₀₀ was greater than the activation observed with either BFR₀ or BFR₁. No activation was observed with BFR₁₀₀₀, for any of the substrates tested, and the rates of hydrolysis of any substrates except imipenem was not affected to any significant degree by an increase in zinc concentration in the assay buffer. However, the presence of zinc in the assay buffer led to reversible inhibition of the carbapenemase activity by this enzyme. The initial experiments shown in Figure 5.1 were conducted on a separate preparation, prepared in a similar manner to BFR₁₀₀₀, and hence showed a similar activity profile. This phenomenon extended to all *Bact. fragilis* CfiA-type metallo- β -lactamases examined in this thesis and to *B. cereus* II, but not to *Sten. maltophilia* L1. Only imipenem hydrolysis could be assayed in these latter two preparations, because both isolates also produced a cephalosporinase which was unable to hydrolyse imipenem [134, 197] but was present during the assays. The L1 metallo- β -lactamase showed slightly greater basal activity when prepared in 1mM ZnSO₄, and slightly less activation than the same culture prepared in lower zinc concentrations. Nevertheless, all *Sten. maltophilia* L1 preparations, (L1₀, L1₁, and L1₁₀₀₀) displayed a similar sigmoidal activation curve as the zinc concentration in the assay buffer was increased. The activation curve was very different from that observed with any of the other metallo- β -lactamases, and of greater amplitude. No significant inhibition of imipenemase activity could be detected at high zinc concentrations, unlike the other metallo- β -lactamases or the serine active site carbapenemase, Nmc-A. These data suggested that the L1 metallo- β -lactamase was not affected by zinc in the same manner as the other carbapenemases. More experiments would be required to determine whether this activation curve is substrate specific, although the complete separation of the L1 metallo- β -lactamase and L2 cephalosporinase in the presence of these three different zinc supplements will be problematic.

A direct mechanistic explanation for this imipenemase inhibition at high zinc concentration is unclear, since the mechanism of β -lactamase hydrolysis by all but the L1 metallo- β -lactamase is not substrate specific.

Cobalt has been successfully substituted for zinc in several metallo- β -lactamases [122, 151], and so the preparations were re-assayed in the presence of increasing cobalt concentrations since Co^{2+} shares a similar ionic radius and charge to zinc [220]. These experiments suggested that the decrease in imipenemase activity of BFR_{1000} at high ZnSO_4 concentrations was not caused by heavy metal poisoning, since no enzyme activation or inhibition could be detected as the cobalt concentration increased [Figure 5.6].

Since this inhibition of hydrolytic activity by increased divalent cation concentration was only noticeable with carbapenems as reporter substrates, the serine active carbapenemase Nmc-A was also tested in a similar manner, but the observation that increasing zinc concentration affected the hydrolysis of nitrocephin and cephaloridine as well as imipenem was consistent with heavy metal poisoning, but inconsistent with that found with the metallo- β -lactamases. Cobalt also affected the imipenem hydrolysis of this β -lactamase reversibly, but no explanation could be found for this.

Heavy metal poisoning would manifest itself as a overall reduction on hydrolytic activity, and it is extremely unlikely that such inhibition would be substrate specific.

It has been proposed that the *B. cereus* II metallo- β -lactamase possesses more than one zinc binding site [116, 122], and it is possible that metallo- β -lactamases from other species may also possess this extra site. The purpose of this additional site is unclear, but the possibility of some form of allosteric enzyme control cannot be discounted.

The implications of these effects on the analysis of suspected metallo- β -lactamases are widespread. It is likely that the presence of zinc ions during preparation has a preserving effect on metallo- β -lactamase activity. This would be consistent with the observations of greater basal activity from BFR_{1000} when compared with BFR_0 and BFR_1 against all substrates, and also the fact that after loss of the co-ordinated metal ion from the active site (for example by ion chelation with EDTA), the addition of zinc cannot fully restore hydrolytic activity. A similar observation was also made for *B. cereus* II by Davies and Abraham [122], and also Benitez *et al.* [118]. It is possible that once the zinc ion has been lost from the active site, the protein becomes denatured in a manner which cannot be totally reversed by the later re-introduction of this ion.

Most workers prepared their suspected metallo- β -lactamases in the absence of a zinc supplement [132, 151] and some have added this ion to the assay buffer [124]. It is thus possible that when assayed, the different metallo- β -lactamases will have been activated to different degrees by this supplement, since not all metallo- β -lactamases showed maximal activation at $100\mu\text{M}$ zinc sulphate [Figure 5.2].

Work in this thesis also demonstrated that the relative hydrolysis rates of various substrates by BFR_0 , BFR_1 and BFR_{1000} are different, suggesting that the substrate profiles of these enzymes will depend partly upon the zinc concentration present during preparation and assaying, as well as the V_{max} and K_m for the substrates in question. This is best shown in Figure 5.1. Assuming a CfiA-type metallo- β -lactamase was prepared with a 1mM ZnSO_4 supplement, the rate of imipenem hydrolysis with a 1mM zinc sulphate supplement in the assay buffer would be approximately 50% of the rate obtained if no zinc sulphate was present during the assay. This would give a misleading result, suggesting that this enzyme could hydrolyse this substrate at only half the rate of cephaloridine, as opposed to a similar rate if the assay was repeated without a zinc supplement in the assay buffer. Whilst this effect is most pronounced with the carbapenems imipenem and meropenem, it also applied to the third generation cephalosporins in a similar manner, but to a lesser degree [Figure 5.1].

Not only are the relative rates of substrate hydrolysis affected by the presence of this ion, but the ID_{50} s of EDTA obtained with enzymes prepared in this manner will vary, by up to a factor of five [Table 5.1].

8.1.3. Recommendations From These Observations.

These observations have widespread ramifications on the purification and assay methodology for these enzymes. Thus I propose that all suspected metallo- β -lactamases be prepared in a suitable zwitterionic buffer with a 1mM zinc (in the form of $ZnSO_4$ or $ZnCl_2$) supplement wherever possible to ensure maximal activity and avoid the problems of metallo- β -lactamase activation. It will not matter whether a metallo- β -lactamase is inhibited by this zinc concentration since this inhibition is reversible, and the supplement could be removed by dialysis at a later stage with no deleterious effects on hydrolytic activity. Initially, such enzymes should be assayed in the absence of any zinc supplement.

Whilst this protocol will suffice for many metallo- β -lactamases including *B. cereus* II, *Sten maltophilia* L1 and CfiA-type enzymes, a detailed study of the effects of zinc on metallo- β -lactamase activity should be undertaken with each enzyme assayed to avoid any of the potentially misleading results described above.

One of the problems associated with fixing the zinc concentration at 1mM during metallo- β -lactamase preparation is that this concentration may not be compatible with certain purification protocols including ion exchange/FPLC, IEF or SDS-PAGE based methods. In such cases, an alternative zinc concentration will have to be added.

8.2. Carbapenems and Clinical Isolates.

8.2.1. *Bacteroides* Group Strains.

Resistance to carbapenem antibiotics amongst members of the *Bact. fragilis* group can be mediated by a variety of mechanisms, as described in Section 1.8. However, the resistance which is mediated by the production of a carbapenem hydrolysing β -lactamase has been shown to result from the expression of a CfiA-type metallo- β -lactamase. The single exception reported to date has been the carbapenemase from a *Bact. distasonis* isolate which appeared not to be a metallo- β -lactamase [157]. The isolates JMS-121, JMS-219 and JMS-221 all produce a single β -lactamase of pI 4.6-5.0. These enzymes have virtually identical substrate and inhibitor profiles to the known CfiA-type metallo- β -lactamase from *Bact. fragilis* ED-262. Kinetic and physical data for these enzymes are also comparable, although there is a discrepancy between the molecular weights determined in this thesis and published values. Several different molecular weights of the CfiA metallo- β -lactamase have been reported by biochemical assays, ranging from 26kDa [216] to 44kDa [152], whereas the molecular weight by DNA analysis and deduced amino acid sequence was found to be 25,249Da [142]. However, all four β -lactamases were purified by FPLC under similar conditions and showed similar elution profiles. For these reasons it can be concluded that these enzymes, characterised from these three isolates, are all CfiA-type metallo- β -lactamases. These strains were originally isolated from geographically distinct regions within Great Britain and from patients who had not received prior imipenem therapy. Since these metallo- β -lactamases could not be transferred to a rifampicin resistant *Bact. fragilis* recipient, it is likely that these enzymes are chromosomally encoded. Transfer to *E. coli* was not attempted since previous work has shown that a known *Bacteroides* plasmid could not be transferred directly to this species. The construction of a specific *Bacteroides* / *E. coli* shuttle vector with an additional origin of replication was required to facilitate plasmid transfer between these two organisms [228]. The antibiograms of these strains [Table 6.1] illustrated that these isolates were found to be resistant to all the antimicrobials tested, including the carbapenems. The

addition of BRL 42715 to imipenem both in a 2:1 ratio and at a fixed concentration of 2mg/l [28, 229] potentiated the antibacterial activity of this carbapenem. When imipenem was tested in a 2:1 ratio with BRL 42715, the MIC of all four strains was lowered by at least two dilutions. However, this combination restored the sensitivity of only JMS-219 by breakpoint. The potential threat from these organisms is great, especially in the case of JMS-121 which was found to be resistant to metronidazole and clindamycin [189] as well as the carbapenems.

It is of concern that strains producing these metallo- β -lactamases are being isolated clinically, and from patients with no prior imipenem therapy. Since these enzymes are capable of hydrolysing virtually every class of β -lactam agent, conferring resistance on the host strain, it is likely that prior treatment with β -lactam compounds other than carbapenems will select for strains producing these enzymes. An increase in the frequency of isolation of these enzymes is inevitable as β -lactam and carbapenem therapy becomes more widespread. The dissemination of these resistance determinants is currently slow, since to date, only one of the *Bacteroides* metallo- β -lactamases has been shown to be plasmid encoded [105].

The sequence of the β -lactamase from *Bact. fragilis* ED-262 has been determined and found to differ from the published CfiA sequence [142] in only 2 amino acids. (A. Fosberry Pers. Comm.). These mutations are not unique, and have also been reported in other CfiA-type metallo- β -lactamases [141, 143]. To date, the sequence of four CfiA-type metallo- β -lactamases have been published, in each case from an isolate identified as *Bact. fragilis*. This number of metallo- β -lactamases have never been identified and sequenced from any other species. These mutations do not appear to affect the catalytic activity of these enzymes, since their substrate and inhibitor profiles are indistinguishable, nor do they alter the physical characteristics including pI or molecular mass by a measurable degree. The sequence identity of all these CfiA-type genes is greater than 98%.

Several mutations have been described in the *B. cereus* II metallo- β -lactamase from *B. cereus* 569, 569H and 5/B/6. These genes show greater heterogeneity, differing by 25 amino acids [121]. These mutations have been shown to affect the hydrolytic properties of this metallo- β -lactamase [135].

8.2.2. Aerobes.

Several of the species examined in this thesis do not frequently cause infections in immunocompetent individuals. Subjects who are at risk from these organisms include immunocompromised or immunosuppressed patients, especially those in ITUs. The most commonly isolated organisms from these patients include *Acinetobacter*, *Klebsiella* and *Stenotrophomonas*. Certain clinical *Acinetobacter* and *Sten. maltophilia* strains are becoming recognised as resistant to a wide range of antimicrobials including the carbapenems, irrespective of whether or not the strains produce an imipenem hydrolysing β -lactamase such as ARI-1 [159] or L1 [151] (this thesis) proving that metallo- β -lactamase production is not a pre-requisite for carbapenem resistance. *Klebsiella* are becoming more difficult to treat in this situation as a result of the rapid dissemination of resistant strains [230-233] and the rapid spread of resistance determinants amongst these organisms, as occurred with the extended spectrum β -lactamases [234-236]. Of the isolates from these species examined in this thesis, only the *Sten. maltophilia* ($n=18$) demonstrated any hydrolytic activity against imipenem. This is consistent with several other reports suggesting that the production of an L1-type metallo- β -lactamase may be ubiquitous in this species. Nevertheless, the strains examined in this thesis from all these species did show a multi-resistant profile with MIC₅₀ values approaching, and MIC₉₀ values above, the recommended breakpoints. The carbapenems and ciprofloxacin showed greatest antibacterial activity, although in several strains, these compounds would probably not be effective *in vivo*. Of great interest however, was the observation that the fourth generation cephalosporin cefepime showed very good activity against *Enterobacter* ($n=9$) and *Klebsiella* ($n=11$); both species showing MIC₉₀s of 1mg/l. The

suggested breakpoint for this compound is 32mg/l [213]. A larger number of strains would be required to determine whether this observation was representative of these species. This compound showed no such activity against the other aerobes examined.

The combination of the β -lactamase inhibitor clavulanic acid with the penicillin amoxycillin did lower the MIC₅₀ and MIC₉₀ values for this compound against all organisms tested. However, this effect was most marked against the random *Bacteroides* isolates examined ($n=166$), where MIC₅₀ and MIC₉₀ values for amoxycillin were 16mg/l and 128mg/l (both above the breakpoint of 4mg/l), but were reduced to 0.5mg/l and 4mg/l respectively in the presence of amoxycillin and clavulanic acid. In this case, the MIC₉₀ for this treatment was below the NCCLS breakpoint of 8mg/l. Cefotaxime was the only cephalosporin with MIC₅₀ values of 8mg/l, below the breakpoint of 32mg/l. These were the only treatments apart from the carbapenems which were effective against these organisms. However, since the carbapenems are currently licensed for use only in ITUs, co-amoxyclav is the only compound available to general practitioners which showed good activity against these organisms with MIC₉₀ values below the breakpoint. The carbapenems imipenem and meropenem showed identical MIC₅₀ and MIC₉₀ values, both at 0.125mg/l and 1mg/l respectively, and were well below the breakpoint of 16mg/l. Of the 166 isolates tested, only 1 organism was found to be resistant to these agents, with MICs of 16mg/l for both compounds. No imipenem hydrolysis could be detected by a cell-free lysate of this organism and thus it was concluded that imipenem resistance amongst these isolates was 0.6%. This value is consistent with other surveys which report the incidence of imipenem resistance between 0.1% and 4% [157, 172]. However, these reports cannot be compared objectively since different groups have used different media and different breakpoints in these surveys, with very few strictly adhering to either the 1991 BSAC [199] or 1992 NCCLS [176] guidelines. It is possible that several of these isolates may harbour a silent metallo- β -lactamase gene [178, 179], and that such a gene could become expressed after repeated exposure to low levels of imipenem.

A conflict has arisen since different guidelines for susceptibility testing have been published by the BSAC [199] and NCCLS [176] which recommend different breakpoints for many compounds. One such example is the recommended breakpoint for amoxycillin and the β -lactam / β -lactamase inhibitor combination amoxycillin / clavulanic acid.

The 1991 BSAC guidelines state that the breakpoint of amoxycillin is 8mg/l and the breakpoint of amoxycillin / clavulanic acid is 1mg/l (against non-*Pseudomonas* aerobes). In this case an eight-fold reduction in MIC in the presence of clavulanic acid would be required purely to prevent an apparent increase in resistance to this 'combination'⁷ treatment as opposed to 'monotherapy'⁸ with amoxycillin. Whilst in certain cases, the presence of clavulanic acid does lower the MIC by this factor, any reduction in MIC mediated by this combination will increase the efficacy of this treatment. In this scenario, it is quite possible for an organism (hypothetical amoxycillin MIC of 4mg/l; mediated by the production of an Ambler class C β -lactamase; resistant to clavulanic acid) to be regarded as resistant to amoxycillin / clavulanic acid, but sensitive to amoxycillin by breakpoint. Such a difference in breakpoint between these two treatments is too large. In addition to this, the BSAC breakpoint for co-amoxycylav is very low, at least two dilutions below any other compound, including the carbapenems, and is not representative of the physiological concentration of this treatment *in vivo*.

The 1992 NCCLS breakpoints of these compounds against aerobes are both 8mg/l. Use of this guideline will better portray the beneficial effect of the presence of the inhibitor clavulanic acid in this treatment.

⁷ Combination therapy in this instance refers to the combination of amoxycillin / clavulanic acid, even though these compounds are administered together in the form of Augmentin. The word combination is used solely to differentiate this treatment from amoxycillin monotherapy.

⁸ Amoxycillin is referred to as monotherapy solely to differentiate this treatment from the combination of amoxycillin and clavulanic acid.

Both of these guidelines recommend the use of IST agar for aerobic organisms, and Wilkins-Chalgren agar supplemented with 5% horse blood for anaerobic organisms. Nevertheless, several groups have determined susceptibilities on other media including DST and Mueller-Hinton agars [174, 237, 238]. The use of these agars is known to affect the MIC values for several organisms [177, 214, 239], with MIC's generally lower on IST than on DST, which are lower than on Mueller-Hinton. This difference was initially attributed to the nutritional value of the media, but other factors including zinc ion concentration has also been implicated in elevating MICs, especially when testing *Sten. maltophilia* isolates [214].

In this thesis, both *P. aeruginosa* ($n=15$) and *Ent. aerogenes* ($n=1$) isolates have been described in which imipenem resistance is mediated not by the production of a metallo- β -lactamase by the hyper-production of a β -lactamase which may belong to Ambler class C, possibly in conjunction with a drop in membrane permeability. Since these β -lactamases could not hydrolyse imipenem, the mechanisms of imipenem resistance were not examined further, as this type of resistance has been previously reported [240-242]. This resistance was found to be stable in the *Ent. aerogenes* isolate; a 26 day passage lowering the MIC of imipenem by only 2 dilutions. The carbapenems were the only β -lactams with MIC₉₀ values within one dilution of the breakpoint, whilst ciprofloxacin was the only compound with MIC₉₀s below the breakpoint of 4mg/l, namely 2mg/l. Whilst infections involving these organisms can be fatal, the multi-resistant profiles of the *Burk. cepacia* strains examined have serious implications for patients suffering from cystic fibrosis. It can be seen from Table 4.3 that these strains were resistant not only to the penicillins and cephalosporins, but resistance was also observed to both carbapenems and to ciprofloxacin. Such organisms are becoming harder to treat in the clinical setting.

8.3. Organisms of the Genus *Flavobacterium*.

Organisms of the genus *Flavobacterium* have been recognised as the causative agent in several infections, most notably neonatal meningitis.

8.3.1. Imipenem Hydrolysis by *Flavobacterium* Isolates.

The 10 *Flavobacterium* strains examined in this thesis were all clinical specimens originally isolated from several different sites [Table 7.1]. All 10 of these were isolated before carbapenem antibiotics were licensed for use clinically. *F. gleum* NCTC 10795 was isolated in 1957, nine years before Sabath and Abraham described the first metallo- β -lactamase [164]. The small scale cultures of all but two of these strains were able to cause significant imipenem hydrolysis. All isolates showed some hydrolytic activity against imipenem. The enzymes responsible for this imipenem hydrolysis would require further examination to determine whether this hydrolysis was mediated by a metallo- β -lactamase or serine active site β -lactamase. The preliminary data [Table 7.2] indicated that these enzymes were sensitive to inhibition with EDTA. The total sample volume present in the spectrophotometric cuvette was 150 μ l; 50 μ l sample, 50 μ l EDTA and 50 μ l substrate. Whilst the EDTA concentration may have been sufficient to effect total zinc sequestration, it is possible that the proteins and ions present in the crude cell extract could overwhelm the EDTA (by total amount) present. If this were the case, true inhibition levels would be greater than those actually observed.

All metallo- β -lactamases reported to date have been shown to be sensitive to EDTA, but the serine active site carbapenemase from *Serratia marcescens* S6 has also been shown to be sensitive, to this chelator [156]. This enzyme was thought to be a metallo- β -lactamase until its sequence was determined [102]. The reason for this inhibition is unclear, but demonstrated that EDTA sensitivity alone is not sufficient evidence to identify unequivocally such an enzyme as a metallo- β -lactamase.

Nevertheless, this does not detract from the fact that imipenem hydrolysis was observed in preparations from all isolates, indicating that all of the tested isolates produced a β -lactamase capable of hydrolysing imipenem. It is possible that all *Flavobacterium* organisms possess a carbapenemase gene, and that production of such a carbapenemase, possibly a metallo- β -lactamase may be ubiquitous in this genus. It is currently thought that carbapenemase production may be ubiquitous in *Aeromonas* spp. [163] and *Sten. maltophilia*. The observations made in this thesis considerably increases the numbers of organisms producing these enzymes. This leads to an increase in the reservoir of β -lactam resistance determinants which could cause treatment failure or could lead to the acquisition of these resistance determinants by more clinically important genera, and hence to treatment failure in cases involving such organisms. It is also conceivable that the presence of such a β -lactamase producing organism could effect treatment failure if colonising the same site as another, more pathogenic organism. It has previously been shown that a metallo- β -lactamase producer could confer protection upon another organism which was present as part of a mixed infection. In an experimental model, a metallo- β -lactamase producing *Bact. fragilis* strain was able to confer resistance upon a β -lactamase negative *E. coli* highly sensitive to β -lactam antibiotics which was colonising the same site [137].

8.3.2. *F. spiritivorum* NCTC 11388.

F. spiritivorum NCTC 11388 and *F. meningosepticum* NCTC 10016 both showed similar β -lactamase profiles by nitrocephin stained IEF, with bands of β -lactamase activity at pI 8.6, 7.6 and 5.4. Whilst cell-free extracts of both isolates were able to hydrolyse imipenem spectrophotometrically and were sensitive to EDTA inhibition [Table 7.2], it was unclear by IEF alone which β -lactamase was responsible for the carbapenem hydrolysis.

F. spiritivorum NCTC 11388 was chosen for further examination as a result of its carbapenem MIC values [Table 7.2] and the fact that one band could still be visualised by nitrocephin-stained IEF after a 100 μ M BRL 42715 overlay [Figure 7.1]. The whole purification methodology for this strain was based upon the deduction that FSP-3 was a metallo- β -lactamase. This deduction proved to be incorrect, once FSP-1 had been resolved and assayed separately from the other enzymes. One of the difficulties encountered with this isolate was the low level of un-inducible β -lactamase production, which made purification problematic. Nevertheless, the carbapenemase FSP-1 exhibited a substrate and inhibitor profile indicative of a metallo- β -lactamase. FSP-2 appeared to be a serine active β -lactamase by its substrate and inhibitor profiles, and may be SHV- or OXA- derived. Only nitrocephin hydrolysis could be unequivocally determined by FSP-3, which remained unaffected by any of the clinical or diagnostic β -lactamase inhibitors tested, including EDTA [Figure 7.7]. It is conceivable that this enzyme may not be a β -lactamase as nitrocephin hydrolysis by proteins other than β -lactamases has been reported previously [243]. An improved separation protocol giving a greater specific activity would be needed to prove this.

The only previous report of a metallo- β -lactamase from an organism of the genus *Flavobacterium* described an enzyme isolated from a strain of *Flavobacterium odoratum* which had a pI of 5.8 [144]. In 1994, Blahova and co-workers [191] reported two *F. meningosepticum* strains able to hydrolyse imipenem. However, both strains were consecutive isolates from the same patient and had identical antibiograms, and may in fact be a single strain. The only other work conducted on these β -lactamases described partial inhibition by EDTA, but as previously stated, this evidence is not sufficient to identify an enzyme as a metallo- β -lactamase. FSP-1, the metallo- β -lactamase described in this thesis from *F. spiritivorum* NCTC 11388 had a pI of 8.6 and a substrate profile distinct from other metallo- β -lactamases described to date [Figure 7.8]. This enzyme is different from the metallo- β -lactamase previously described from an isolate of this genus, and suggests that the carbapenemases produced by organisms of the genus *Flavobacterium* are heterogeneous in nature, more so that the

carbapenemases described from *A. hydrophila*, *Bact. fragilis* or *B. cereus* [Table 1.4]. It is likely that the *F. odoratum* metallo- β -lactamase and this enzyme are not closely related.

From this it can be concluded that the FSP-1 β -lactamase is novel and is probably a novel metallo- β -lactamase.

CHAPTER NINE

Epilogue

Bacterial resistance to carbapenem antibiotics is rare. Of the possible resistance mechanisms, the production of a carbapenemase is the most common, and represents the greatest threat to the clinical use of these compounds. Both serine active carbapenemases and metallo- β -lactamases have been described from a variety of species. In reports where novel carbapenemases have been described, little or no work has been undertaken to optimise the purification methodology applied to these enzymes. In addition to this, many workers have simply applied an existing assay system to the analysis of these enzymes, but added a zinc sulphate supplement to increase metallo- β -lactamase activity, without considering any possible inhibitory effect of this supplement on the hydrolytic activity of these enzymes. Work undertaken in this thesis redresses this balance and also characterises the substrate specific inhibitory effect of zinc on the hydrolytic activity of several different carbapenemases, both serine active site β -lactamases and metallo- β -lactamases. This thesis also describes an assay system to determine the most effective ion exchange matrix to purify a β -lactamase.

Three imipenem resistant *Bact. fragilis* isolates were found to produce a metallo- β -lactamase which showed biochemical properties indicative of a CfiA type metallo- β -lactamase, but did not appear to be transferable. The novel metallo- β -lactamase FSP-1, from a clinical isolate of *F. spiritivorum* was characterised, and other data presented in this thesis indicate that carbapenemase production may be ubiquitous within this genus, suggesting that carbapenemase production is more common than previously thought. This greatly increases the reservoir of potential carbapenem resistance determinants which could potentially lead to treatment failure.

CHAPTER TEN

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Correspondence

Prophylaxis after splenectomy

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Sir,

We read with interest the leading article by Read & Finch (1994), which reflected current interest by concentrating on the susceptibility of splenectomised patients to overwhelming sepsis caused by *Streptococcus pneumoniae* (Kinnersley, Wilkinson & Srinivasan, 1993; McMullin & Johnston, 1993). However it should be pointed out, that in one study by Ellison & Fabri (1983) *S. pneumoniae* was responsible for only half of all infections after splenectomy. Other capsulated organisms are also possible causes of severe infection, especially in children and young adults. Attention needs to be drawn to the possibility of fatal infection caused by *Haemophilus influenzae* type b.

This problem was recently met in a young, previously fit man aged 19 years who had had his spleen removed, because of an accident in 1989. One evening in December 1993, he complained of feeling unwell and of having 'flu' like symptoms. At about 5 a.m. the following morning, when seen by his mother, he said he had slept badly and felt sick. She left the house and on her return at about 3 p.m. found him dead. Post-mortem swabs of meninges, nose and upper trachea were taken, together with pericardial fluid, heart blood and CSF. All specimens yielded a profuse growth of *H. influenzae* type b.

We believe that this case, like the one reported by Teare & O'Riordan (1992), reinforces the case for the administration of *H. influenzae* type b vaccine not just to all splenectomised children who have not yet received it, but also to all splenectomised adults (Flegg, 1994).

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Effects of zinc concentration on the imipenemase activity of a metallo- β -lactamase from a *Bacteroides fragilis* clinical isolate

J Antimicrob Chemother 1994; 34: 295–297

Sir,

The expression of a serine active chromosomal β -lactamase by organisms from the *Bacteroides fragilis* group is common, conferring resistance to several penicillins and cephalosporins. However, the production of a metallo- β -lactamase is currently thought to represent the main threat to the use of carbapenems for serious anaerobic clinical infections. It has been reported that bacteria from this group can produce a variety of metallo- β -lactamases which have the ability to hydrolyse carbapenems as well as cephalosporins and penicillins (Cuchural *et al.*, 1990; Rasmussen, Gluzman & Tally, 1991).

We report an observation made during an assay for β -lactamase activity of an enzyme produced by a *B. fragilis* clinical isolate designated JMS-221. This isolate was originally isolated at Ninewells hospital, Dundee, from the abdominal wounds of a patient with myelofibrosis and received from Dr J. Brazier (Public Health Laboratory, Cardiff). This isolate was positively identified as *B. fragilis* by API 32A.

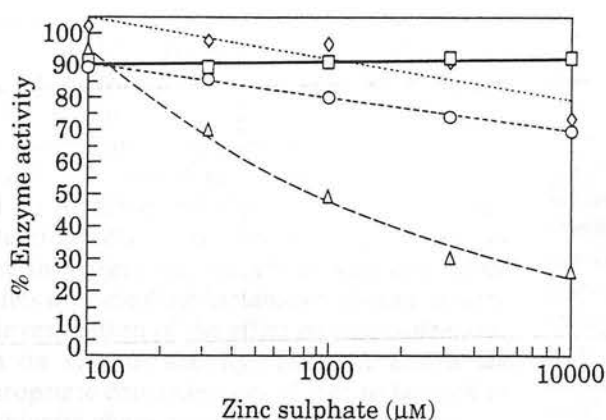


Figure. The effect of zinc sulphate supplements on the activity of the metallo- β -lactamase produced by JMS-221 for various substrates. \square , Cephaloridine; \diamond , cefuroxime; \circ , cefotaxime; \triangle imipenem.

Crude sonicated cell extracts containing the enzyme were found to be sensitive to overnight dialysis against 25 mM PIPES buffer-pH 7.0 supplemented with 10 mM EDTA. This dialysis led to greater than 99% inhibition of the enzyme with imipenem and nitrocefin as substrates, but had no effect on the activity of the TEM-1 β -lactamase. The activity of the *B. fragilis* enzyme was restored to 22% of the original activity by a 10 min pre-incubation of the EDTA treated enzyme with 10 mM zinc sulphate which, when it was diluted, resulted in a final concentration of 167 μ M in the assay solution. As a result of this observation, and the fact that this enzyme was able to hydrolyse imipenem, it is proposed that this enzyme is a metallo- β -lactamase.

The enzyme was partially purified by passing a sonicated, cell free extract through a Sephadex G75 gel filtration column, with 25 mM PIPES buffer pH 7.0 supplemented with 1 mM zinc sulphate as the elution buffer. During all stages of the preparation, 25 mM PIPES buffer pH 7.0 supplemented with 1 mM zinc sulphate was used as a buffer as it is known that metallo- β -lactamases require zinc to maintain enzyme activity (Sabath & Abraham, 1966; Davies & Abraham, 1974; Felici *et al.*, 1993). The molecular weight of the enzyme determined by this gel filtration was 25,000. Prior to the assay for activity, the enzyme was dialysed four times against a greater than 100-fold excess of PIPES buffer made up in MilliQ water without the zinc sulphate supplement to remove any excess Zn^{2+} ions which were not co-ordinated with the enzyme.

The assay for β -lactamase activity was conducted at 37°C on a Perkin-Elmer Lambda 2 spectrophotometer in 25 mM PIPES buffer pH 7.0 made up in MilliQ water, measuring the decrease in absorbance of 100 μ M substrate. Cephaloridine, cefuroxime, cefotaxime, and imipenem were used as substrates, all at 100 μ M and the decrease in absorbance was measured at 255, 260, 265 and 299 nm respectively. It has been reported that the type of glassware used during the preparation and storage steps can greatly affect the concentration of zinc in the assay solution (Hawkey *et al.*, 1993). To circumvent this, disposable glassware was used which had been rinsed in MilliQ de-ionized water, and all bottles had plastic caps.

During the assay for β -lactamase activity with imipenem as substrate, it was observed that the concentration of zinc sulphate present in the assay buffer affected the rate of hydrolysis of imipenem. By extending this observation, it was possible to determine an ID_{50} of zinc sulphate (concentration required to inhibit enzyme activity by 50%) for this enzyme, taking the rate of hydrolysis of 100 μ M imipenem without any added zinc sulphate as 100%. This ID_{50} was determined as 1 mM zinc sulphate as shown in the figure, which also shows the enzyme activity against cephaloridine, cefuroxime, and cefotaxime at different concentrations of zinc sulphate. In each case, the rate of hydrolysis of 100 μ M substrate without added zinc sulphate was taken as 100% enzyme activity. It was found that the effect of zinc sulphate concentration on the activity of this metallo- β -lactamase was significantly greater for imipenem than for any of the cephalosporins tested.

The addition of zinc sulphate to the assay buffer did not cause any detectable hydrolysis of the β -lactam ring of any of the substrates in our assay system despite the fact that β -lactam hydrolysis by Zn^{2+} ions has been reported when present alone or in conjunction with a buffer (Benitez *et al.*, 1991). A 5 min pre-incubation of the enzyme with the assay buffer containing the appropriate zinc concentration made negligible difference to the assay as compared with no pre-incubation. It was also noted that in the experiments with and without pre-incubation, the addition of less than 10 μ M zinc sulphate increased the rate of hydrolysis of imipenem to marginally over 100% (data not shown) indicating that this may be a more appropriate concentration for this assay.

This work details the identification of a

metallo- β -lactamase in *B. fragilis*. We have made the novel observation that the concentration of Zn^{2+} in the assay buffer affects the rate of hydrolysis of imipenem to a significantly greater extent than the hydrolysis of cephalosporins. Additional research is required to comprehend fully this observation and to ascertain whether it applies to other metallo- β -lactamases. As a consequence of these investigations, we advise that any initial studies on metallo- β -lactamases should include an investigation of the effect of zinc concentration on enzyme activity. This will enable an appropriate concentration of zinc to be used in the kinetic characterization of the enzyme.

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Susceptibility of enterococci to ciprofloxacin

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Sir,

Ciprofloxacin is widely regarded as having only modest antibacterial activity against enterococci. Guidelines produced by the British Society for Antimicrobial Chemotherapy (BSAC) Working Party (1991) recommend breakpoint values of > 4 mg/L to define resistance and ≤ 1 mg/L to indicate susceptibility. As a consequence of this a high proportion of enterococci are typically found to be of moderate susceptibility to ciprofloxacin i.e. $\text{MIC} > 1 < 4$ mg/L, (Barry & Jones, 1989). In our laboratory we tested 136 consecutive clinical isolates of enterococci from a variety of sources against ciprofloxacin. Stepwise dilutions of ciprofloxacin were prepared in Diagnostic Sensitivity Test agar (DST) (Unipath, Basingstoke, UK) and an inoculum of 10^5 organisms was applied to the agar surface. Disc testing was also performed in parallel for all strains on DST agar using standard methods. Two strengths of disc content were used containing 1 and 5 μg ciprofloxacin respectively (Mast Laboratories, Bootle, UK). The results of the MIC studies are shown in the Table.

The results indicate that this particular population of enterococci showed a marked bimodal distribution of sensitive and highly resistant strains as no single isolate demonstrated a MIC of between 4 and > 8 mg/L of ciprofloxacin. When these MIC results were then compared with antibiotic zone sizes, it was found that, perhaps not surprisingly, almost all of the enterococci in the "moderately susceptible" group (66% of the total isolates) showed virtually no inhibition to a ciprofloxacin 1 μg disc. However, when a ciprofloxacin 5 μg disc was tested there was excellent discrimination between moderately susceptible strains (all isolates produced a zone size ≥ 15 mm) and resistant isolates which showed no zone of inhibition. We believe these findings demonstrate that ciprofloxacin 1 μg disc provides little discrimination when testing enterococci and a 5 μg disc produces more informative results.

We would wish to emphasize that we do not advocate ciprofloxacin as a drug of choice against enterococci, particularly in serious infections. However, there is some evidence that it may be clinically appropriate, for example, in cases of complicated urinary tract infection (Muranaka & Greenwood, 1988). Indeed, perhaps the greatest value of testing